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ANALYTICAL CHEMISTRY ORGANIC CHEMISTRY BIOLOGICAL CHEMISTRY
FOOD & NUTRITION MICROBIOLOGY & FERMENTATION INDUSTRY
SOIL & FERTILIZER

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 1, p. 1~12, 1960]

Studies on Browning Reactions between Sugars and Amino Compounds*

Part V. Isolation and Characterization of New Carbonyl Compounds, 3-Deoxy-osones formed from N-Glycosides and their Significance for Browning Reaction

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Received April 6, 1959

Both aliphatic and aromatic amine-N-pentosides decompose to form melanoidins by weak acid catalysis. From their reaction mixture, an unknown carbonyl compound, $C_{17}H_{16}N_8O_{10}$, has been isolated as its bis-2,4-dinitrophenylhydrazone. And, similarly from the reaction mixture of either aliphatic or aromatic amine-N-D-glucoside, another unknown hydrazone, $C_{18}H_{18}N_8O_{11}$, has been isolated. As the results of characterization, it has been proved that the former is 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone and the latter is 3-deoxy-D-glucosone bis-2,4-dinitrophenylhydrazone.

It has been shown that 3-deoxy-osone is the intermediate in the melanoidin formation. Furthermore, its significance in the browning reaction is pointed out.

In the previous paper of this series[1,2], it has been concluded that aromatic amine-N-xylosides produce red pigments, melanoidins, and furfural which is not an intermediate in melanoidin formation, consequently, there is another active compound except furfural, and that aromatic amine-N-glucosides produce melanoidins and 5-hydroxymethylfurfural (HMF) which forms melanoidin, but HMF is only a part of origin of melanoidins formed from N-glucoside. In this paper, another active compound except furfurals is investigated.

Many investigators have shown that browning reactions are blocked by the addition of various sorts of carbonyl reagents[3~6]. It has been found that aliphatic amine-N-glycoside, especially pentoside, rapidly produces melanoidin in the presence of acetic acid. The reaction is blocked almost completely by the addition of hydroxylamine, semicarbazide, dimedone or barbituric acid as shown in Table I. This fact shows that in this browning reaction the carbonyl compound is an intermediate. An attempt to isolate this unstable carbonyl compound as its stable

TABLE I. INHIBITION OF BROWNING REACTION OF N-D-XYLOSYL-NORMAL-BUTYLAMINE BY THE ADDITION OF CARBONYL REAGENTS.

An amount of 1.64 g of N-D-xylosyl-*n*-butylamine was dissolved in 1 ml of methanol and to this solution 0.5 ml of glacial acetic acid was added and then the crystal of carbonyl reagent was added and heated at 60° for ten minutes, and diluted to 0.002M.

carbonyl reagent added	molecular equivalent	$\log I_0/I$ (450 mμ)
none	—	0.368
$NH_2OH \cdot HCl$	1	0.00
$NH_2CONHNH_2 \cdot HCl$ $+CH_3COONa$	each 1	0.00
dimedone	1	0.074
〃	2	0.003
barbituric acid	1	0.008

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* Presented at the Symposium entitled "Amino-Carbonyl Reaction Connected with Foodstuffs" of the Annual Meeting of the Agricultural Chemical Society of Japan, Tokyo, April 8, 1959.

1) Part III of this series: This Bulletin, **22**, 85 (1958).

2) Part IV: This Bulletin, **23**, 551 (1959).

3) J. E. Hodge, *J. Agr. Food Chem.*, **1**, 928 (1953).

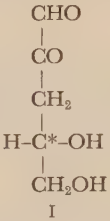
4) H. M. Barnes and C. W. Kaufman, *Ind. Eng. Chem.*, **39**, 1167 (1947).

5) J. E. Hodge and C. E. Rist, *J. Am. Chem. Soc.*, **75**, 316 (1953).

6) E. R. Stadman, "Advances in Food Research", Vol. 1, p. 325 (1948).

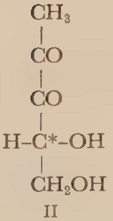
derivative has been carried out. The 2, 4-dinitrophenylhydrazine (2, 4-DPH) reagent which precipitates carbonyl compounds almost completely at room temperature was selected. To the methanol solution (2M) of N-D-xylosyl-*n*-butylamine, a molecular equivalent of glacial acetic acid was added, and the solution was warmed at 55° for various minutes. To each of the resulting brown solutions, a 2, 4-DPH reagent was added. The precipitate of hydrazones obtained from the above each brown solution warmed within forty minutes was treated by the procedure described in part III[1] of this series in order to isolate furfural 2, 4-dinitrophenylhydrazone, but this could not be obtained at all (Table II). This fact shows that furfural does not play any role under the condition described above. From the fraction of the precipitate which was adsorbed by alumina (Brockmann) in ethylacetate and eluted with methanol, an unknown hydrazone, $C_{17}H_{16}N_8O_{10}$, m.p. 257° (dec.), was isolated in pure crystalline form. When *p*-nitrophenylhydrazine was employed instead of 2, 4-DPH, the corresponding hydrazone, $C_{17}H_{18}N_6O_6$, m.p. 261° (dec.), was isolated. When the relation between the yield of this hydrazone and melanoidin formation was traced, as shown in Fig. 1, it became clear that this carbonyl compound is an intermediate in melanoidin formation. Accordingly, the chemical structure of this unknown hydrazone was investigated.

The hydrazone mentioned above gave a violet color in the alcoholic sodium hydroxide test[7,8], which showed it to be bis-2, 4-dinitrophenylhydrazone of α-dicarbonyl compound. It gave diacetate in a mixture of pyridine and acetic anhydride at room temperature and consumed one molecular equivalent of periodic acid to produce formaldehyde. From these experimental results, the chemical structure of this α-dicarbonyl compound is concluded to be I, II, or III of the following structures.



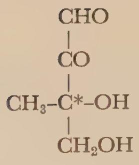
$$\begin{array}{c} CHO \\ | \\ CO \\ | \\ CH_2 \\ | \\ H\text{-}C^*\text{-}OH \\ | \\ CH_2OH \end{array}$$

I



$$\begin{array}{c} CH_3 \\ | \\ CO \\ | \\ CO \\ | \\ H\text{-}C^*\text{-}OH \\ | \\ CH_2OH \end{array}$$

II



$$\begin{array}{c} CHO \\ | \\ CO \\ | \\ CH_3\text{-}C^*\text{-}OH \\ | \\ CH_2OH \end{array}$$

III

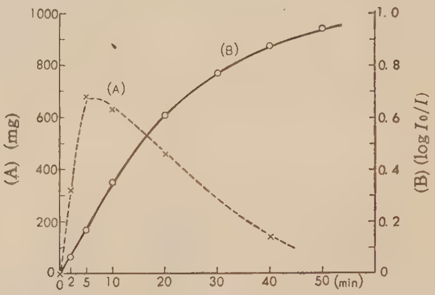


Fig. 1. Correlation between Formation of 3-Deoxy-D-xylosone and Melanoidin Formation.

(A) Milligram of 3-deoxy-D-xylosone bis-2,4-dinitrophenylhydrazone obtained, when 6.0 g of D-xylose and 3.6 g of *n*-butylamine in 5 ml of methanol were heated at 60~65° for fifteen minutes and cooled and then to this solution 2.5 ml of glacial acetic acid was added and heated at 55° for various times, and the resulting brown solution was treated by the procedure described in experimental part.

(B) Log I_0/I at 450 mμ, when 6.0 g of D-xylose and 3.6 g of *n*-butylamine in 5 ml of methanol were heated at 60~65° for fifteen minutes and cooled and then to this solution 2.5 ml of glacial acetic acid was added and heated at 55° for various times and diluted to 0.002M solution.

An exactly identical compound, $C_{17}H_{16}N_8O_{10}$, m.p. 257° (dec.), was isolated from D-arabinose plus *n*-butylamine under the same condition. When this crystal and that from N-D-xylosyl-*n*-butylamine were mixed and recrystallized from methanol, the crystal thus obtained was quite identical with both of the original crystals in their infrared spectra. Then, the same compound, m.p. 257° (dec.), was also isolated from L-arabinose plus *n*-butylamine. This crystal acted in a different manner. Namely, when the crystal from L-arabinose and that from D-xyloside were mixed in a quantity of each 25 mg and recrystallized from methanol, the resulting crystal obtained in a yield of 45 mg changed in its crystal-form and melted at 254° (dec.) and showed a distinct difference in its infrared spectrum (Fig. 2). Therefore, this compound has one optically active center, and the crystal from

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[7] C. Neuberg and E. Strauss, *Arch. Biochem.*, **7**, 211 (1945).

[8] M. Keeney, *Anal. Chem.*, **29**, 1489 (1957).

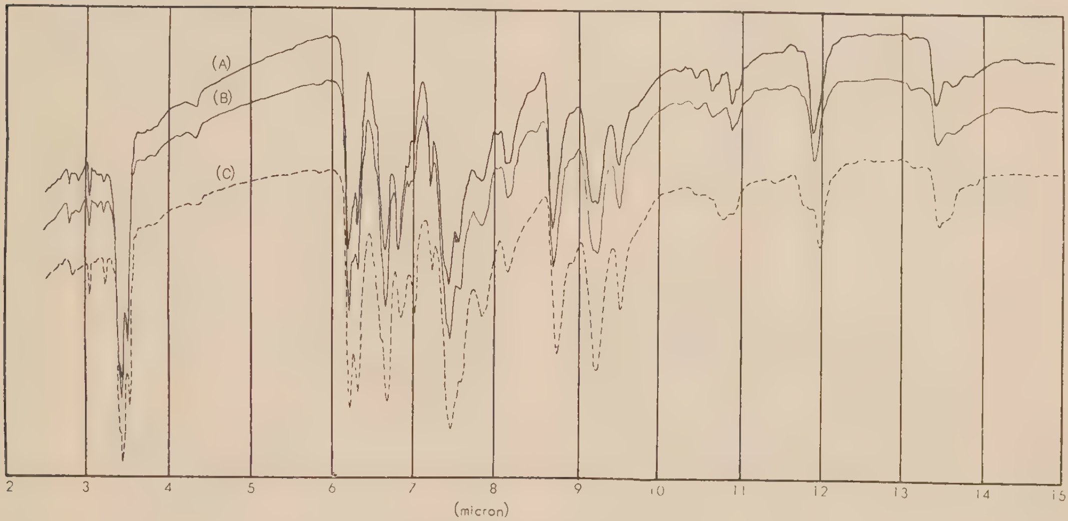

FIG. 2. Infrared Spectra of

(A) 3-Deoxy-D-xylosone bis-2,4-dinitrophenylhydrazone, obtained from N-D-xylosyl-*n*-butylamine,
(B) 3-Deoxy-L-xylosone bis-2,4-dinitrophenylhydrazone, obtained from L-arabinose plus *n*-butylamine,
(C) 3-Deoxy-D,L-xylosone bis-2,4-dinitrophenylhydrazone, obtained by recrystallization of the mixture of (A) and (B).

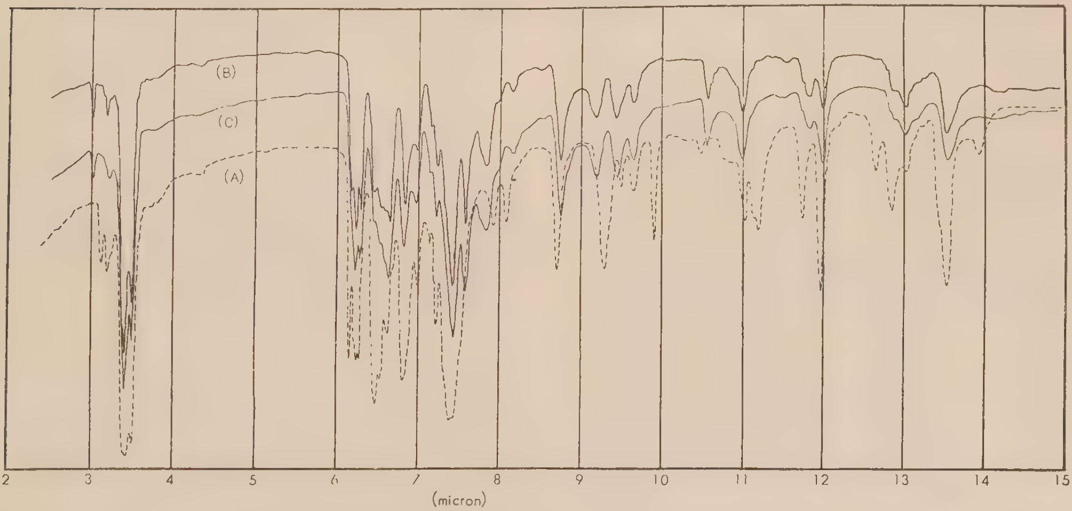

FIG. 3. Infrared Spectra of Periodic Acid Oxidation Product of 3-Deoxy-xylosone Bis-2, 4-dinitrophenylhydrazone.

(A) Syn-form.
(B) Anti-form, obtained from 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone.
(C) Anti-form, obtained from 3-deoxy-D-glucosone bis-2,4-dinitrophenylhydrazone.

D-xylose and that from L-arabinose are related to be D- and L-configurations respectively, and further, the active center is located at the C 4 position of sugar, in consideration of the steric configurations of xylose and arabinose. Accordingly, structure III is eliminated not only on account of the asymmetric carbon which may be racemic in the structure III but also on account of two readily acetylable hydroxyl groups which may not be tertiary.

When this bis-2, 4-dinitrophenylhydrazone was oxidated by periodic acid at room temperature, it afforded two monohydrazones, m.p. 245° and 270°, respectively which gave a reddish brown color with alcoholic sodium hydroxide[7,8]. Either the former (m.p. 245°) or the latter (m.p. 270°) gave a mixture consisting of them, when it was refluxed in acetic acid. Namely, they are stereoisomers mutually and the crystal of m.p. 245° is assigned as syn-form on account of the shift of the N–H streching absorption in its infrared spectrum and the crystal of m.p. 270° is assigned as anti-form (Fig. 3)[9,10]. This monohydrazone gave a molecular formula, $C_{16}H_{10}N_8O_8$. The infrared spectra of both syn- and anti-forms showed neither carbonyl nor hydroxyl band (Fig. 3). Eight oxygen atoms of the molecular formula, $C_{16}H_{10}N_8O_8$, are counted for oxygens of the four nitro-groups belonging to two 2, 4-dinitrophenyl residues. Consequently, there is no oxygen attached to the original carbon chain of sugar. Karrer and Pfaehler[11], and Chargaff and Magasanik[12] studied on periodic acid oxidation of glucose phenylosazone and isolated 1-phenyl-4-phenylhydrazono-pyrazolone-5 (IV) as the oxidation product (Fig. 4). In this case, one oxygen atom attached to the carbon chain of sugar remains as C=O in the pyrazolone ring. If bis-2, 4-dinitrophenylhydrazone of structure II is oxidated by periodic acid, the oxidation product is considered to be 3-methyl-substituted pyrazolone derivative, as an analogue of oxidation of glucose phenylosazone, which is impossible to lose the above oxygen in the pyrazolone ring. Accordingly, the structure II is also eliminated. When bis-2, 4-dinitrophenylhydrazone of structure I is oxidated by periodic acid, as shown in Fig. 5, the oxidation product is capable of losing the last one oxygen attached to the sugar chain by dehydration on account of the presence of adjacent methylene group. Thus, the unknown bishydrazone, $C_{17}H_{16}N_8O_{10}$, is bis-2, 4-dinitrophenylhydrazone of 3-deoxy-xylosone (V), and its oxidation product, $C_{16}H_{10}N_8O_8$, is either VII or VIII (Fig. 5).

To the methanol solution (2M) of N-D-glucosyl-*n*-butylamine, an equal mole of glacial acetic acid was added, and the solution was warmed at 55° for one hour. To the resulting brown solution, 2, 4-DPH reagent was added. From the precipitate of hydrazones, an unknown hydrazone, $C_{18}H_{18}N_8O_{11}$, m.p. 252° (dec.), was isolated as the crystal having one mole of ethylacetate as the solvate, but HMF 2, 4-dinitrophenylhydrazone was not obtained at all. This

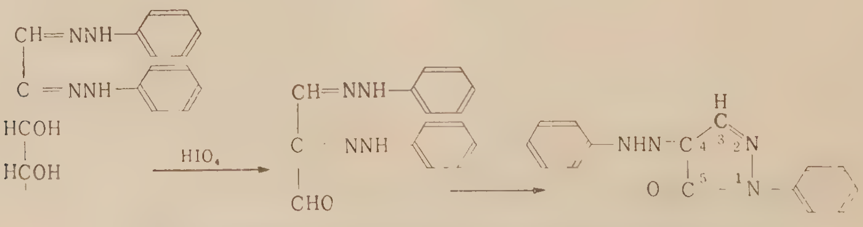

FIG. 4. Periodic Acid Oxidation of Glucose Phenylosazone.

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[9] L. I. Braddock, et al., *Anal. Chem.*, **25**, 301 (1953).
[10] F. Ramirez and A. F. Kirby, *J. Am. Chem. Soc.*, **76**, 1037 (1954).
[11] P. Karrer and K. Pfaehler, *Helv. Chim. Acta*, **17**, 766 (1934).
[12] E. Chargaff and B. Magasanik, *J. Am. Chem. Soc.*, **69**, 1459 (1947).

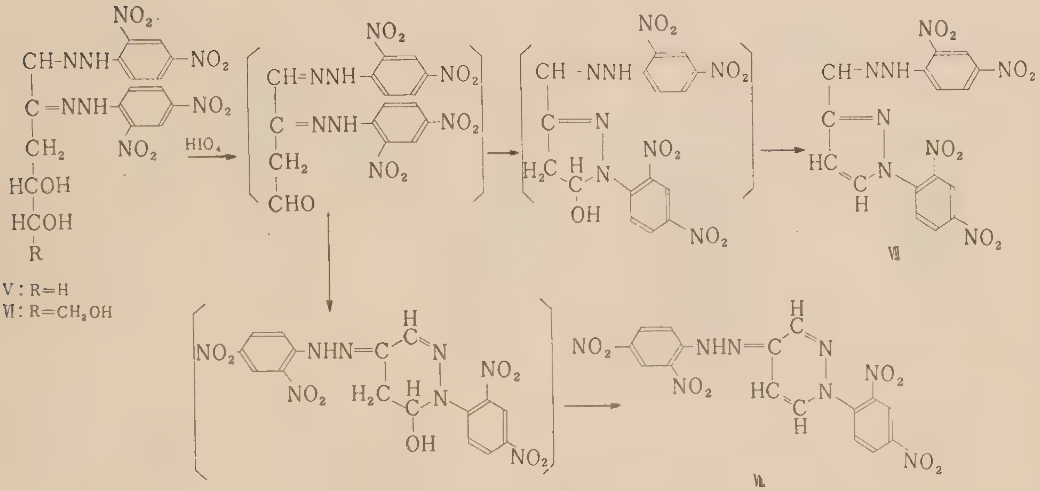

FIG. 5. Periodic Acid Oxidation of 3-Deoxyosone Bis-2,4-dinitrophenylhydrazone.

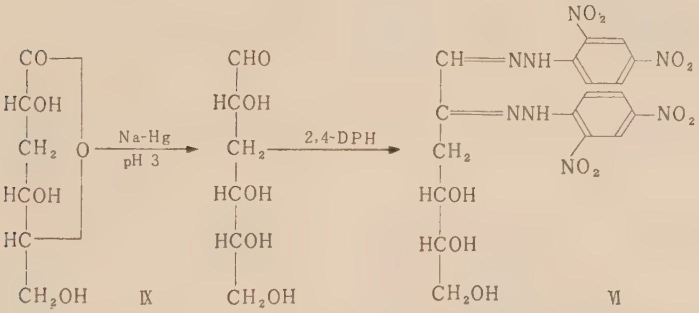

FIG. 6. Synthesis of 3-Deoxy-D-glucosone Bis-2,4-dinitrophenylhydrazone.

fact shows that HMF does not play any role under the condition described above. And further, from N-D-glucosyl-*p*-aminobenzoic acid (N-D-glucosyl-PABA) in methanolic hydrogen chloride under the condition described in part IV[2], the same hydrazone, $C_{18}H_{18}N_8O_{11}$, m.p. 252° (dec.), was isolated, besides HMF 2, 4-dinitrophenylhydrazone. This compound also gave a violet color in the alcoholic sodium hydroxide test[8] which showed it to be α-bis-2, 4-dinitrophenylhydrazone. It gave triacetate in a mixture of pyridine and acetic anhydride at room temperature and consumed two moles of periodic acid to yield formaldehyde and the same compound, $C_{16}H_{10}N_8O_8$, with the oxidation product of 3-deoxy-xylosone bis-2, 4-dinitrophenylhydrazone (Fig. 3). Therefore, this unknown hydrazone is 3-deoxy-D-glucosone bis-2, 4-dinitrophenylhydrazone (VI). This compound has been synthesized as follows: The mixture of α- and β-D-dextro-metasaccharins (IX)[13] was reduced with sodium amalgam at pH 3[14] to form 3-deoxy-sugar. The syrup of 3-deoxy-sugar was made to react with 2, 4-DPH reagent at room temperature for a long period. Osazone formation gradually occurred to produce VI (Fig. 6).

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[13] J. C. Sowden, *J. Am. Chem. Soc.*, **76**, 3541 (1954).

[14] N. Sperber, H. E. Zaugg and U. M. Sandstrom, *ibid.*, **69**, 915 (1947).

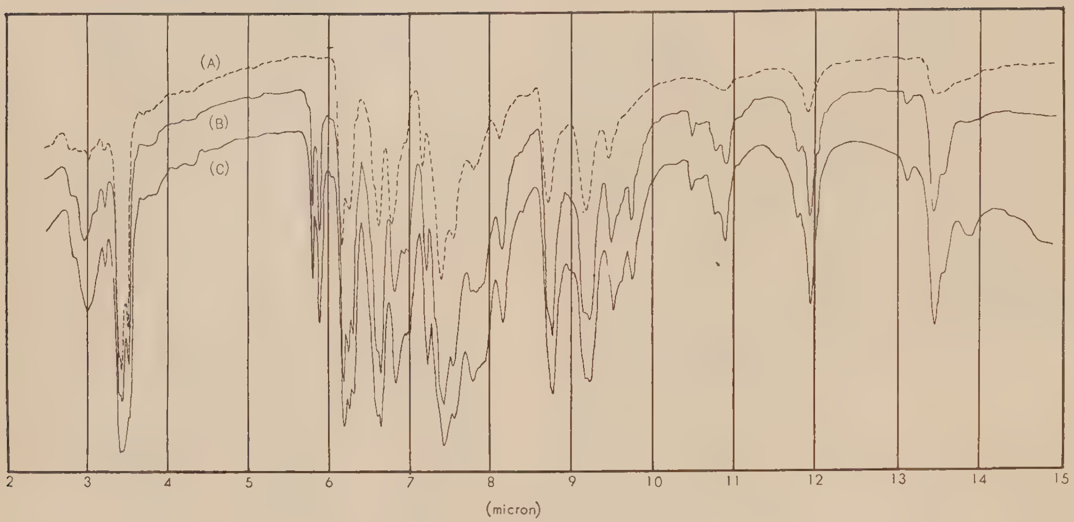

FIG. 7. Infrared Spectra of

(A) 3-Deoxy-D-glucosone Bis-2,4-dinitrophenylhydrazone,
(B) (A) with Ethylacetate of Crystallization, isolated from Reaction Mixture of N-D-Glucosyl-*n*-butylamine,
(C) (A) with Ethylacetate of Crystallization, synthesized from D-Dextro-metasaccharins.

TABLE II. FORMATION OF FURFURALS, 3-DEOXYOSONES AND MELANOIDINS UNDER VARIOUS CONDITIONS.

	reacting substance	weight(g)*	solvent	concn. (M)	temp. (°C)	time	$\log I_0/I$ (600 mμ)**	(A)or(B) (mg)***	(C)or(D) (mg)***
1.	D-xylose+glycine	1.2+0.6	H_2O(pH 6.5)	each 2	80	20 min.	0.051	2(A)	51(C)
2.	N-D-xylosyl-*n*-butylamine	1.64	2N CH_3COOH-MeOH	2	60	10 min.	0.183	0	82(C)
3.	〃	〃	2N CH_3COOH-H_2O (pH 6.5)	2	60	60 min.	0.046	0	48(C)
4.	〃	〃	0.5N HCl-MeOH	0.5	27	24 hrs.	0.002	0	0
5.	N-D-xylosyl-aniline	1.80	2N CH_3COOH-MeOH	2	60	10 min.	0.007	4(A)	80(C)
6.	〃	〃	0.5N HCl-MeOH	0.5	27	24 hrs.	0.458	230(A)	0
7.	N-D-xylosyl-PABA	2.15	0.2N HCl-MeOH	0.2	27	24 hrs.	0.389	414(A)	14(C)
8.	N-D-glucosyl-*n*-butylamine	2.02	2N CH_3COOH-MeOH	2	55	60 min.	0.032	0	39(D)
9.	N-D-glucosyl-PABA	2.53	0.5N HCl-MeOH	0.5	25	72 hrs.	0.542	81(B)	84(D)

* All of them are 0.008 M*.
** Diluted to 0.005 M solution.
*** (A) Furfural 2,4-dinitrophenylhydrazone[1].
(B) 5-Hydroxymethylfurfural 2,4-dinitrophenylhydrazone[2].
(C) 3-Deoxy-D-xylosone bis-2,4-dinitrophenylhydrazone.
(D) 3-Deoxy-D-glucosone bis-2,4-dinitrophenylhydrazone.

DISCUSSION

As shown in Table II, 3-deoxyosone is formed from various reacting substances under acid catalytic condition, and therefore 3-deoxyosone seems to be a common intermediate in browning reactions. Wolfrom, et al. studied on the browning reaction between glycine and xylose in aqueous solution under refluxing for two hours and detected furfural as its 2,4-dinitrophenylhydrazone[15]. Also, in the present work, from

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[15] M. L. Wolfrom, R. D. Schuetz and L. F. Cavalieri, *J. Am. Chem. Soc.*, **71**, 3518 (1949).

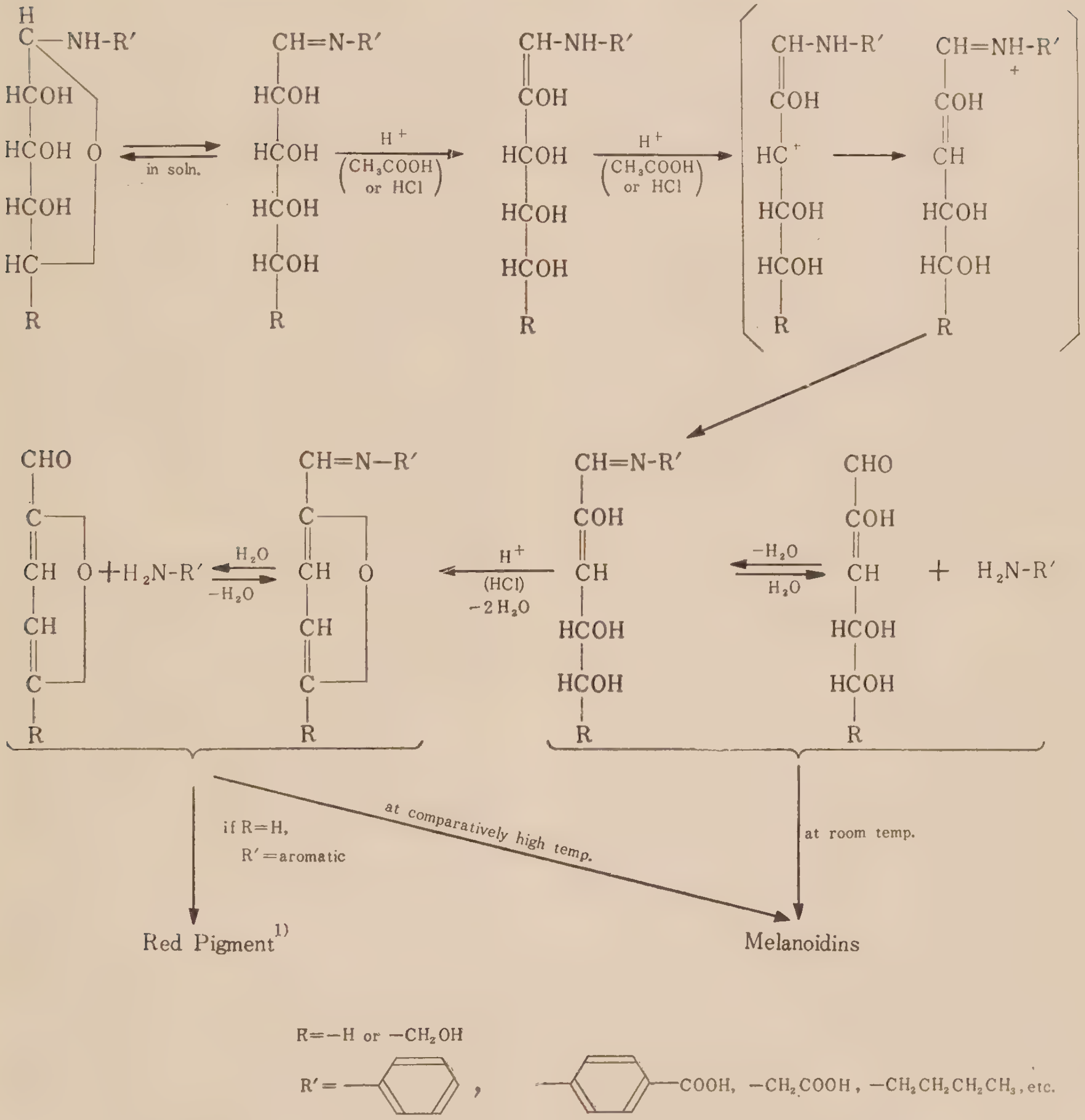

FIG. 8. Formation Mechanism of 3-Deoxyosones, Furfurals and Melanoidins from N-Glycosides.

the reacting solution of glycine and D-xylose (2M aqueous solution of each at pH 6.5) at 80° for twenty minutes, a trace of furfural 2,4-dinitrophenylhydrazone was detected. However, an exceedingly larger amount of 3-deoxyosone was detected in spite of its unstability (Fig. 1) as shown in Table II. In addition, in the case of N-D-xylosyl-*n*-butylamine, as described above, furfural was not detected at all. These facts indicate the important significance of 3-deoxyosone in browning mechanism, namely, 3-deoxyosones reacting with amine, form melanoidins more readily rather than they form furfurals. From N-D-xylosyl-PABA in methanolic hydrogen chloride at room temperature, a considerable amount of furfural (20 %) was formed1), but only a small amount of 3-deoxyosone was detected (Table II). In this case it is considered that 3-deoxyosone formed from N-xyloside produces furfural on account of strong acidity of hydrogen chloride and a part of 3-deoxyosone reacts with amine to produce melanoidin. The browning mechanism of N-glycoside under acid catalytic condition is summarized in Fig. 8. The mechanism of formation of 3-deoxyosone from N-glycoside is well explained by 1, 2-enolization of N-glycoside caused by the action of proton.

3-Deoxyosone is a typical α-dicarbonyl compound, which reacts with α-amino acid to cause Strecker degradation[16]. Although the Strecker degradation is a non-essential reaction for melanoidin formation[3], it is an important reaction for flavor formation and carbon dioxide production of foodstuffs in which the role of 3-deoxyosone should not be overlooked.

EXPERIMENTAL

All melting points were uncorrected. The 2,4-DPH solution was prepared by Shriner's method[17]. Spectrophotometric measurements were carried out with a Hitachi Photo-Electric Spectrophotometer in methanol solution. Infrared spectra were measured by a Kōken DS-301 Infrared Spectrophotometer in Nujol mull.

Preparation of N-D-Xylosyl-*n*-butylamine[18]. A mixture of 12 g of finely powdered D-xylose (Merck), 5.9 g of *n*-butylamine and 10 ml of methanol was heated at 60～65° with stirring for fifteen minutes. After heating, the flask was cooled with water and allowed to stand at room temperature, and then crystallization soon occurred to form a lump of crystal. After crystallization had been completed, the lump of crystal was triturated with 20 ml of absolute ethanol, the crystal was subsequently filtered with suction and washed with a mixture of absolute ethanol and ether (1 : 1 by volume) and dried. The yield was 8.4 g of needles (51%, m.p. 92～94°). Recrystallization from absolute ethanol gave 4.5 g, m.p. 95～97°. This substance readily became tarry, even when it was placed in a desiccator with calcium chloride. *Anal.* Calcd. for $C_9H_{19}NO_4$: C, 52.66; H, 9.33; N, 6.83%. Found: C, 53.01; H, 9.35; N, 6.86%.

Preparation of N-D-Glucosyl-*n*-butylamine (Based on Pigman[19]). A mixture of 18 g of finely powdered D-glucose, 7.2 g of *n*-butylamine and 10 ml of methanol was heated at 60～65° with stirring for twenty minutes. To the resulting yellow viscous solution, 60 ml of hot 94% ethanol was added and allowed to stand at room temperature, and crystallization occcurred. In this case, when absolute ethanol was used instead of 94% ethanol, crystallization did not occur, and the addition of 2.5 ml of water caused the solution to crystallize. This fact indicates that the crystal of N-D-glucosyl-*n*-butylamine is hydrated. After being allowed to stand for one day at room temperature, the crystal was filtered, washed with ether and dried. The yield was 17.5 g of needles, m.p. 89～91° (69%). *Anal.* Calcd. for $C_{10}H_{21}NO_5 \cdot H_2O$: C, 47.40; 9.15; N, 5.53%. Found: C, 47.60; H, 9.41; N, 5.47%.

Isolation of 3-Deoxy-xylosone as its 2,4-Dinitrophenylhydrazone.

(1). (a). From D-xylose plus *n*-butylamine.

A mixture of 6.0 g of finely powdered D-xylose, 3.6 g of *n*-butylamine and 5 ml of methanol was heated at 60° with stirring for fifteen minutes. Within about ten minutes D-xylose completely dissolved. The flask containing the resulting solution was cooled with water and then 2.5 ml of glacial acetic acid was added and immediately warmed up to 55° and maintained at this temperature for exactly ten minutes. The reaction mixture rapidly became brown. Then, the flask was immediately cooled with water and 2,4-DPH (2.0 g) solution[17] was added. To this mixture, 50 ml of water was step by step added and it was allowed to stand at room temperature for two hours. The precipitate was filtered, washed with 50% ethanol and dried. The weight was 1.21 g. This mixed powder of hydrazones was extracted with ethylacetate repeatedly (ca. 300 ml), and filtered. The filtrate was charged on a column (diameter, 3 cm) of alumina (70 g, Brockmann) packed with ethylacetate. After feeding, the column was washed with ethylacetate in an amount as much as a five-fold-volume of the alumina packed, and then eluted with a mixture of methanol and ethylacetate (1 : 9 by volume). About 500 ml of the eluate was collected and concentrated to 30 ml under reduced pressure to crystallize out. The crystalline powder was filtered, washed with methanol and dried. An amount of 625 mg of fine orange rods, m.p. 256° (dec.), was obtained. Recrystallization was attained in the following manner. The crystal was dissolved in a large volume of hot ethylacetate—methanol (1 : 1 by volume) and concentrated to a small volume under reduced pressure to obtain a pure crystal, m.p. 257° (dec.), soluble in ethylacetate and alcohol, insoluble in water and benzene. *Anal.* Calcd. for $C_{17}H_{16}N_8O_{10}$: C, 41.47 ; H, 3.28 ; N, 22.76 %. Found : C, 41.41 ; H, 3.41 ; N, 22.67%. Determination of molecular weight (Rast). Calcd. for $C_{17}H_{16}N_8O_{10}$: 492. Found: 444. λ_{max} (in methanol): 434 mμ (ε, 4.72×10^4).

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[16] A. Schoenberg, R. Moubacher and A. Mostafa, *J. Chem. Soc.*, **1948**, 176.

[17] R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds", p. 171 (1948).

[18] E. Votoček and F. Valentin, *Collection Czechoslov. Chem. Communs.*, **6**, 77 (1934).

[19] W. Pigman, E. A. Cleveland, D. H. Couch and J. H. Cleveland, *J. Am. Chem. Soc.*, **73**, 1976 (1951).

(b). From N-D-xylosyl-*n*-butylamine.

A quantity of 1.64 g of freshly prepared N-D-xylosyl-*n*-butylamine was dissolved in 1 ml of methanol, and to this solution 0.5 ml of glacial acetic acid was added and warmed at 55° for ten minutes. The subsequent procedure was exactly the same as (1) (a) performed in a scale of one-fifth of it. The yield was 123 mg, m.p. 256° (dec.).

(2). From D-arabinose plus *n*-butylamine.

One gram of D-arabinose and 0.6 g of *n*-butylamine were used and treated in quite the same manner as (1) (a), in a scale of one-sixth. The yield was 86 mg, m.p. 256° (dec.).

(3). From L-arabinose plus *n*-butylamine.

Three grams of L-arabinose and 1.8 g of *n*-butylamine were used and treated in quite the same way as (1) (a), in a scale of one half. The yield was 274 mg, m.p. 256° (dec.).

Isolation of 3-Deoxy-xylosone as its *p*-Nitrophenylhydrazone. A mixture of 6.0 g of D-xylose, 3.6 g of *n*-butylamine and 5 ml of methanol was heated at 65° with stirring for ten minutes. The flask containing the reacting solution was cooled with water, and 2.5 ml of glacial acetic acid was added and it was then immediately warmed at 55° for exactly ten minutes. The flask containing the resulting brown solution was cooled with water,and after a solution of *p*-nitrophenylhydrazine (3.0 g) in a mixture of 95% ethanol (100 ml) and 3 N hydrochloric acid (50 ml) was added, 50 ml of water was added to this mixture and allowed to stand at room temperature for two hours. The precipitate was filtered, washed with 50% ethanol and dried. The weight was 1.41 g. This powder of hydrazones was extracted with about 500 ml of hot ethylacetate and filtered off and the filtrate was allowed to cool at room temperature and charged on a column (diameter, 3 cm) of alumina (80 g) packed with ethylacetate. After feeding, the column was washed with ethylacetate in an amount as much as a three-fold-volume of the alumina packed. After washing, the brown top-part of the alumina column was removed off in a depth of about 1 cm and the lower orange part was eluted with a mixture of methanol and ethylacetate (1 : 1 by volume). About 1 liter was employed for elution. This eluate was concentrated to 10 ml under reduced pressure. During concentration, crystallization occurred. Thirty milliliters of ethylacetate was added to it and the crystal was triturated, filtered off, washed with ethylacetate and dried. An amount of 695 mg of red fine needles, m.p. 259° (dec.), was obtained. When the needles were recrystallized by dissolution in a mixture of methanol and ethylacetate (1 : 1 by volume) and by concentration to a small volume under reduced pressure, a pure crystal, m.p. 261° (dec.), was obtained, soluble in ethylacetate and alcohol, insoluble in water and benzene. *Anal.* Calcd. for $C_{17}H_{16}N_6O_6$: C, 50.74; H, 4.51 ; N, 20.89 %. Found : C, 50.52 ; H, 4.50 ; N, 20.49%. λ_{max} (in methanol) : 438 mμ (ε, 7.29×10^4).

When the heating condition after the addition of glacial acetic acid described above was at 60° for ten minutes, the yield was 400 mg, m.p. 259° (dec.), and in case of heating at 50° for ten minutes, the yield was 600 mg, m.p. 259° (dec.).

Preparation of Diacetate of 3-Deoxy-xylosone Bis-2,4-dinitrophenylhydrazone. One hundred milligrams of 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone was dissolved in 2 ml of pyridine and 0.8 ml of acetic anhydride was added and allowed to stand at room temperature for twenty-four hours. The reacting solution was poured into about 100 ml of ice water and allowed to stand at room temperature. After dissolution of ice, the resulting suspension was filtered and the precipitate was washed with water and dried. This was dissolved in benzene, and charged to adsorb with a column (diameter, 1.2 cm) of alumina (15 g) packed with benzene. After adsorption, the column was washed with benzene and then eluted with benzene containing ethylacetate up to 10% by volume. The eluate from the first yellowish band was discarded and the next eluate from the main brown band was collected and dried up slowly under reduced pressure to obtain yellow needles. However, this crystal contained ethylacetate as the solvate and showed double melting points of 79° and 187°. When this was either heated in vacuum or recrystallized from methanol, the crystal readily lost the ethylacetate of crystallization. The yield of the crystal recrystallized from methanol was 69 mg (59%), m.p. 188~189°. *Anal.* Calcd. for $C_{21}H_{20}N_8O_{12}$: C, 43.75 ; H, 3.50 ;

N, 19.44%. Found: C, 43.94; H, 3.59; N, 19.38%. Determination of acetyl-group (Kuhn-Roth): Calcd. for $C_{17}H_{14}N_8O_{10}\cdot 2(COCH_3)$: 14.88%. Found: 14.53%.

Measurement of Consumption of Periodic Acid. (Based on Akiya[20]) Twenty-five milligrams of the sample was dissolved in 8 ml of dioxane, and to this solution 3 ml of the aqueous or methanol solution of periodic acid (ca. 850 mg of $HIO_4\cdot 2H_2O$ was dissolved per 50 ml) was added and allowed to stand at room temperature for twenty-four hours. An amount of 4 ml of this reacting solution was pipetted, and 10 ml of water and 10 ml of a borate–borax buffer solution (3 g of boric acid and 4 g of borax was dissolved per 100 ml of water) and 0.2 g of potassium iodide were added and then the iodine educed was titrated by N/10 arsenious acid solution adjusted to pH 7 with sodium hydroxide. This titration value corresponds to the periodic acid unreacted; consequently, the difference between the blank value and this titration value corresponds to the periodic acid consumed. If this difference is $\varDelta$ ml, the molecular equivalent of consumed periodic acid, x, can be calculated by the following equation:

$$x=\varDelta\,\mathrm{ml}\times\frac{11}{4}\times\frac{1}{10}\times\frac{\text{molecular weight of sample}}{0.025}\times\frac{114}{1000}\times\frac{1}{228}$$

Periodic Acid Oxidation of 3-Deoxy-xylosone Bis-2,4-dinitrophenylhydrazone.

(1). Measurement of consumption of periodic acid.

3-Deoxy-xylosone bis-2,4-dinitrophenylhydrazone treated by the procedure described above consumed 0.94 molecular equivalent (m.e.) in a dioxane—methanol solution and 1.06 m.e. in a dioxane—water solution.

(2). Formation of formaldehyde.

Fifty milligrams of the sample was dissolved in 8 ml of dioxane and to this solution 3 ml of aqueous solution of periodic acid dihydrate (50 mg) was added and allowed to stand at room temperature for twenty-four hours. And then five milliliters of N/10 arsenious acid solution (pH 7) was added and it was then treated with steam distillation. An amount of 20 ml of the distillate was taken and poured into 50 ml of an aqueous solution of dimedone (100 mg) and heated in a water bath for thirty minutes and allowed to stand at room temperature overnight. The crystal crystallized out was filtered and dried. The yield was 12 mg, m.p. 187°. Upon admixture with an authentic formaldimedone, the melting point remained unchanged.

(3). Isolation of the oxidation product.

One hundred milligrams of the sample was dissolved in 14 ml of dioxane, and to this solution 6 ml of aqueous solution of periodic acid dihydrate (100 mg) was added and allowed to stand at 30° for twenty-four hours. The reacting solution was poured into 200 ml of water, and when precipitation had been completed, it was filtered, washed with water and dried. This yellow powder was dissolved in benzene and charged on a column (diameter, 1.2 cm) of alumina (15 g) packed with benzene. The fraction which passed through the column without adsorption was collected and dried up. Light yellow needles were obtained in a yield of 12 mg, m.p. 245° (syn-form), $\lambda_{\max}$ (in methanol), 251 mμ (ε, 2.59×10^4) and 376 mμ (ε, 2.92×10^4). Further, the column was eluted with a mixture of benzene and ethylacetate (9 : 1 by volume). When this eluate was dried up, yellow needles were obtained in a yield of 69 mg, m.p. 270° (anti-form), $\lambda_{\max}$ (in methanol), 382 mμ (ε, 3.25×10^4). When either syn- or anti-form was refluxed in glacial acetic acid, it gave a mixture of them which could be separated by chromatography described above**. *Anal.* Calcd. for $C_{16}H_{10}N_8O_8$: C, 43.45; H, 2.28; N, 25.34%. Found: C, 44.17; H, 2.07; N, 24.90%.

Isolation of 3-Deoxy-D-glucosone as its 2,4-Dinitrophenylhydrazone.

(1). From N-D-glucosyl-*n*-butylamine in methanol—acetic acid.

A mixture of 10.1 g of freshly prepared N-D-glucosyl-*n*-butylamine, 5 ml of methanol and 2.5 ml of glacial acetic acid was warmed at 55° for sixty minutes. The reacting solution gradually became brown. After warming, the flask was cooled with water and 2,4-DPH (2.0 g) solution[17] was added to it and allowed to stand at room temperature for two hours. The resulting precipitate was filtered, washed with 50% ethanol and dried. The weight was 350 mg. This powder of hydrazone was extracted with 80 ml of ethylacetate and filtered. When the filtrate was allowed to stand in a refrigerator overnight, yellow needles appeared. The crystal was filtered, washed with a small amount of ethylacetate and dried at room temperature. An amount of 85 mg, m.p. 252° (dec.), was obtained. Furthermore, the mother liquor and washings were collected and concentrated to 40 ml under reduced pressure and held in a refrigerator overnight to crystallize out. The second crop was obtained in an amount of 110 mg, m.p. 252° (dec.). The infrared spectrum of this crystal showed carbonyl bands (Fig. 7), and when the crystal was heated at 100° in vacuum, the bands disappeared. Accordingly, the crystal contains ethylacetate as the solvate. Determination of ethylacetate of crystallization: The crystal (m. p. 252°, dec.) was weighed after drying in a desiccator with sulfuric acid in vacuum for three days. Then, it was dried at 100° in vacuum in phosphorous pentoxide for seven hours. Percentage of the loss in weight was calculated. Calcd. for $C_{18}H_{18}N_8O_{11}\cdot CH_3COOC_2H_5$: 14.4%. Found: 13.3%. The crystal without ethylacetate, m.p. 252° (dec.), was analysed. *Anal.* Calcd. for $C_{18}H_{18}N_8O_{11}$: C, 41.38; H, 3.47; N, 21.45%. Found: C, 41.73; H, 3.49; N, 21.89%. Determination of molecular weight (Rast): Calcd. for $C_{18}H_{18}N_8O_{11}$: 522. Found: 539. λ_{max} (in methanol), 434 mμ (ε, 4.73×10^4).

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[20] S. Akiya, *J. Pharm. Soc. Japan*, **71**, 867 (1951).

** Some lots of alumina (Brockmann) adsorbed both syn- and anti-forms in benzene, in which case a large amount of benzene was allowed to flow through the column, and then the band of syn-form and that of anti-form were separated.

(2). From N-D-glucosyl-PABA in methanolic hydrogen chloride.

A quantity of 12.7 g of N-D-glucosyl-PABA was suspended in 80 ml of methanolic hydrogen chloride (0.5 N) and stood at 25° for seventy-two hours. N-D-Glucosyl-PABA gradually dissolved and the reacting solution became dark brown. Then 2,4-DPH (2.0 g) solution[17] was added to it and further, 50 ml of water was added slowly and allowed to stand at room temperature for two hours. The resulting precipitate was filtered, washed with 50% ethanol and dried. The weight was 3.12 g. This powder of hydrazones was extracted with 500 ml of ethylacetate and filtered. The filtrate was allowed to stand in a refrigerator overnight to crystallize out. The yellow needles educed were filtered and washed with a small amount of ethylacetate and dried at room temperature. The yield was 305 mg, m.p. 252° (dec.). The mother liquor and washings were collected and concentrated to 100 ml under reduced pressure and allowed to stand in a refrigerator overnight. The second crop was 115 mg, m.p. 249° (dec.).

Preparation of Triacetate of 3-Deoxy-D-glucosone Bis-2,4-dinitrophenylhydrazone. One hundred milligrams of 3-deoxy-D-glucosone bis-2,4-dinitrophenylhydrazone (dried at 100° in vacuum) was dissolved in 2 ml of pyridine and, to this solution 0.8 ml of acetic anhydride was added and allowed to stand at room temperature for twenty hours. The reacting solution was poured into 100 ml of ice water. After the dissolution of ice, the precipitate was filtered, washed with water and dried. This yellow powder was dissolved in benzene and charged to adsorb with a column (diameter, 1.2 cm) of alumina (15 g) packed with benzene. After adsorption, the column was washed with benzene and then eluted with a mixture of benzene and ethylacetate (4 : 1 by volume). The fraction of the first eluted brown band was collected and dried up. The crystal obtained was washed with methanol and dried. The yield was 84 mg (68%), m.p. 174~175°, of orange platelets. *Anal.* Calcd. for $C_{24}H_{24}N_8O_{14}$: C, 44.45; H, 3.73; N, 17.28%. Found: C, 44.95; H, 3.78; N, 17.25%. Determination of acetyl-group (Kuhn-Roth): Calcd. for $C_{18}H_{15}N_8O_{11}\cdot 3(COCH_3)$: 19.91%. Found: 19.28%.

Periodic Acid Oxidation of 3-Deoxy-D-glucosone Bis-2,4-dinitrophenylhydrazone.

(1). Measurement of consumption of periodic acid.

3-Deoxy-D-glucosone bis-2,4-dinitrophenylhydrazone treated by the procedure described above consumed 1.96 m.e. of periodic acid in dioxane—methanol solution and 2.04 m.e. in dioxane—water solution.

(2). Formation of formaldehyde.

Fifty milligrams of the sample was treated by the same procedure as in the case of 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone. The yield of formaldimedone was 10 mg, m.p. 187°.

(3). Isolation of oxidation product.

One hundred milligrams of the sample was dissolved in 14 ml of dioxane and to this solution 6 ml of aqueous solution of periodic acid dihydrate (200 mg) was added and it was allowed to stand at 30° for twenty-four hours. The reacting solution was poured into 200 ml of water. When precipitation had been completed, the precipitate was filtered, washed with water and dried. This yellow powder was treated with the same adsorption chromatography as in the case of the oxidation product of 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone. An amount of 8 mg, m.p. 245°, of light yellow needles was obtained. This crystal was the same compound as the syn-form of the hydrazone obtained by periodic acid oxidation of 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone on account of both infrared spectra and mixed melting point. And further, 62 mg, m.p. 270°, of yellow needles was obtained from the fraction of the anti-form of the hydrazone. The infrared spectrum of this crystal showed little difference from that of the anti-form obtained from 3-deoxy-xylosone bis-2,4-dinitrophenylhydrazone in spite of non-depression of mixed melting point. Then, the same chromatography was repeated for this crystal, and the fraction of the first-half of the eluate by a mixture of benzene and ethylacetate (9 : 1 by volume) was dried up. The infrared spectrum of this fraction was quite identical with that of the anti-form obtained from 3-deoxy-xylosone bis-2, 4-dinitrophenylhydrazone (Fig. 3).

Synthesis of 3-Deoxy-D-glucosone Bis-2,4-dinitrophenylhydrazone.

A mixture of α- and β-D-dextro-metasaccharins was prepared in accordance with Sowden's method[13] from D-glucose by the action of sodium hydroxide. It was then reduced by Sperber's method[14] with sodium amalgam at pH 3, in order to prepare the syrup of a mixture of 3-deoxy-D-glucose and 3-deoxy-D-mannose. An amount of 0.5 g of this syrup was dissolved in 5 ml of water, and to this solution a 2,4-DPH (0.8 g) solution[17] was added and allowed to stand at room temperature for twenty-four hours. The resulting precipitate which contained impurities was filtered off with suction and further, the filtrate was allowed to stand at room temperature for two weeks. Then orange needles gradually crystallized out. The crystal was filtered, washed with 50% ethanol and dried. Recrystallization from ethylacetate gave 65 mg, m.p. 250° (dec.), of yellow needles. The infrared spectrum of this compound was identical with that of 3-deoxy-D-glucosone bis-2,4-dinitrophenylhydrazone obtained from N-D-glucoside described above (Fig. 7) and its melting point was not depressed on admixture.

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Polarographic Studies on Sarkomycin

Part II. Polarographic Determination of Sarkomycin

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Sarkomycin can be assayed by adsorption on Duolite S-30 Resinous Adsorbent below pH 2, elution with methanol, dilution of the eluate with citric acid solution containing sodium chloride (pH 2.0), and estimation of the reducing current of the first wave by polarography.

Sarkomycin can now be assayed by means of a microbiological method. The microbiological assay requires a long time and furthermore, involves a fairly large error. Kotake et al.[1] have reported on a spectrophotometric method based on the red colorlization occurring by reaction with anthrone in sulfuric acid and proposed the name "anthrone value" for the obtained, because degradation products of sarkomycin are similarly colorized and the sample is over-estimated. The author[2] has reported that the reducing currents of sarkomycin are proportional to the concentrations in only the same lot of sarkomycin; but as an aqueous solution of sarkomycin decreases its microbiological activity out of proportion to the decrease in the reducing currents and the polarographic method is not applicable for its determination without pre-treatment. This may be caused by the fact that some fractions, whose reducing currents are not proportional to the microbiological activities, or some impurities, showing a half-wave potential similar to sarkomycin, exist in neutral extracts of sarkomycin as shown in the countercurrent distribution. The author et al.[3] have obtained a good result in the purification process using Duolite S–30 Resinous Adsorbent: Sarkomycin was adsorbed on Duolite S–30 in a strong acid region below pH 2 and eluted with methanol. The yield, microbiologically, was 82.67% on the average with a standard deviation of 2.06%. Methanol elution separates sarkomycin from impure substances such as the colored substances. The resin used is regenerated by rinsing with alkali and subsequently with acid. This purification enables the polarographic determination of sarkomycin.

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[1] Y. Kotake, S. Kondô and K. Miyagi, *J. Antibiotics* (Japan), Ser. B, **8**, 45-7 (1955).

[2] K. Ida, This Bulletin, **23**, 40-5 (1959).

EXPERIMENTAL

Apparatus:

Polarograph. A "Yanagimoto" Model PS–51 Polarograph equipped with a "Yanagimoto" Model PR–1 Polarorecorder was employed and the dropping mercury electrode used here had a value of $m^{2/3}\ t^{1/6}$ of 1.71 $mg^{2/3}\ sec.^{1/6}$ The temperature was maintained constant at 21°C during electrolysis.

Resin column. The column was prepared from a glass tubing which was 30 cm in length and 15 mm in diameter. One end of the tube provided with a porous sintered-glass disk was drawn out to a narrow tube affixed to a rubber tube, 3 cm in length, and fitted with a glass tip and a pinch clamp.

Reagents:

Solutions were prepared from reagent grade chemicals but standardization was not necessary.

Sodium hydroxide, 4% by volume.

Hydrochloric acid, 4% by volume, 0.1 N, and 0.01 N.

Methanol, absolute.

Citric acid solution. A quantity of 21.014 g of citric acid and 11.69 g of sodium chloride was dissolved in distilled water, the pH adjusted to 2.0 with hydrochloric acid, and the solution diluted to 1 liter.

Preparation of resin column:

Twenty ml of wet Duolite S–30 (Chemical Process Co.) was placed in the resin column. The resin bed was loosened and freed from air bubbles by inverting and shaking the column. The column was regenerated before each use by rinsing with 20 ml of 4% sodium hydroxide and subsequent 20 ml of 4% hydrochloric acid. It was unnecessary to wash with water because sarkomycin was adsorbed at strong acid pH.

Determination procedure:

Sarkomycin aqueous solution (25–500 units* in total) was diluted to approximately 20 ml (below pH 2) with 0.1 N hydrochloric acid and passed through the resin column at a space velocity of 4∼5. The column was rinsed with 0.01 N hydrochloric acid and then the adsorbed sarkomycin was eluted with methanol at the same velocity. The first 5∼10 ml aliquot of the eluate containing no methanol and therefore no sarkomycin was discarded. The subsequent eluate was collected into a 50-ml volumetric flask holding 25 ml of the citric acid solution, until it was filled. After being bubbled with hydrogen or nitrogen gas in a electrolytic cell to remove the dissolved oxygen, the resulting solution was polarographed from −0.5 v to −1.5 v. Sarkomycin was determined by comparing the obtained reducing current of the first wave with a standard curve, prepared by the same procedure using a standard sarkomycin.

DISCUSSION

An impure substance was observed from the results of countercurrent distribution, previously reported[2], showing a half wave potential similar to that of the second wave of sarkomycin and having no microbiological activity. On the other hand, the reducing currents of the second wave of sarkomycin were not proportional to the microbiological activities of some fractions, though those of the first wave were almost proportional to microbiological activities. Besides, the author et al.[4] reported on the isolation of pyruvic acid as an impure substance in sarkomycin acid oil, showing another half-wave potential at pH 2.0 and half-wave potentials similar to those of the first of sarkomycin above pH 3.0. In consideration of these facts, it is appropriate to utilize the first wave below pH

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[3] T. Hara, K. Ida, Y. Yamada and H. Umezawa, Japan Patent 10698 (1957).

* The potency "unit" in this report is equivalent to the microbiological potency of mg*, assigned in the Minimum Requirements of Antibiotic Products published by the Japanese Government.

[4] T. Hara, H. Yamada, K. Ida and T. Yamada, *J. Antibiotics* (Japan), Ser. B, **9**, 184∼8 (1956).

2.0 for polarographic determination of sarkomycin.

The standard curve obtained by the above-described procedure using a standard sarkomycin solution is shown in Fig. 1 and its correlation analysis is given in Table I.

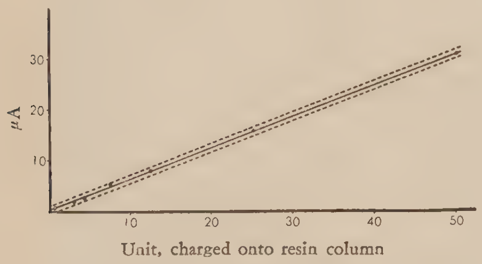

FIG. 1. Calibration Curve

TABLE I. CORRELATION ANALYSIS

Coefficient of correlation	r=0.99
Regression of x upon y	$x=1.624y-0.158$ (x=total unit of adsorbed sarkomycin and y=reducing current in μA)
Standard error	0.837

The sarkomycin aqueous solution left for 1 week lost its microbiological activity rapidly—its microbiological activity fell to approximately 55% at 0°C and to approximately 12% at 20°C. Nevertheless, its reducing currents by polarography without pre-treatment decreased only to approximately 81% at 0°C and to approximately 36% at 20°C[2]. From these facts it is obvious that the decrease rates in microbiological activities are not in agreement with those of reducing currents and therefore sarkomycin cannot be determined from a standard curve without pre-treatment on account of no proportionality between the microbiological activities and reducing currents. However the values of preserved sarkomycin solutions and other sample lots determined by this procedure agreed with the microbiological activities as shown in Table II. Values obtained by the anthrone method are described therewith, but degradation products were similarly colored by anthrone so that the obtained anthrone values were higher than the values obtained by the microbiological assay method.

Futhermore, amorphous sodium salts of sarkomycin gave a reduction wave of impurity before those of sarkomycin appeared, but this was not observed in the sarkomycin solution treated with Duolite S-30 as Fig. 2.

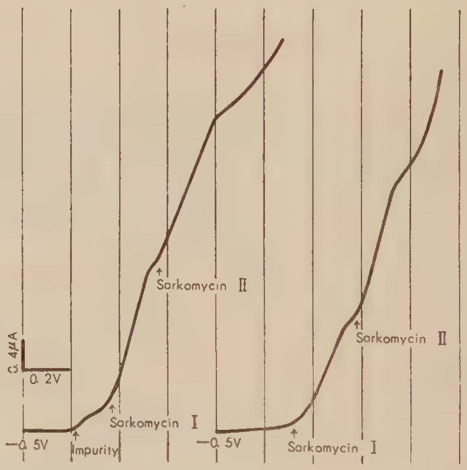

FIG. 2. Polarograms of sarkomycin without and with pre-treatment, pH 2.0

Left. No treatment
Right. After treatment with Duolite S-30

TABLE II. DETERMINATION EXAMPLES OF SARKOMYCIN.

No.	Microbiological assay	Polarographic assay	Anthrone assay	Remarks
Sample 1	370 unit/ml	365 unit/ml	420 unit/ml	Aqueous solution of sodium salt
Sample 2	100 unit/ml	112 unit/ml	154 unit/ml	After preservation of sample 1 at 4°C for 10 days
Sample 3	50 unit/ml	45 unit/ml	98 unit/ml	After preservation of sample 1 at 4°C for 25 days
Sample 4	1.1 unit/mg	1.2 unit/mg	1.2 unit/mg	Amorphous sodium salt

TABLE III. INFLUENCE OF METHANOL CONCENTRATION ON REDUCING CURRENT.

No.	Reducing current of the first wave	Citric acid solution	Added distilled water	Remarks
Run 1	3.2 μA	5 ml	0 ml	Diluted to 10 ml with methanol
Run 2	3.4	5	0.5	〃
Run 3	3.5	5	1.0	〃
Run 4	4.6	5	5.0	No addition of methanol

Sarkomycin aqueous solution (100 units/ml) was diluted 50 times with citric acid solution and 5 ml aliquots of the resulting solution were taken into four 10-ml volumetric flasks, followed by the addition of 0, 0.5, 1.0, and 5 ml of distilled water and subsequent addition up to the mark. The reducing currents by polarography are given in Table III and the addition of methanol diminished the reducing currents of the first wave, but there was no significant deviation among runs 1∼3.

TABLE IV. INFLUENCE OF pH ON REDUCING CURRENT.

pH	Reducing current of the first wave
1.8	6.2 μA
2.0	6.0
2.4	5.9
3.0	6.4

The reducing currents by polarography of sarkomycin aqueous solutions diluted 10 times with citric acid solutions, which were previously adjusted the pH to 1.8, 2.0, 2.4, and 3.0 with 0.1 N hydrochloric acid and 0.1 N sodium hydroxide, are given in Table IV and no significant deviation is observed among them.

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Enzymatic Properties of Bacterial α-Amylase reactivated with Various Alkaline Earth Metals

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Bacterial α-amylase previously inactivated by the removal of calcium was reactivated by the addition of not only calcium salts but also other alkaline earth metal salts, the degree of reactivation depending on the kind of the metal added. The enzymatic properties of the amylase thus reactivated with those metals were investigated comparatively. The calcium-reactivated amylase was far more stable than amylases reactivated by other metals. The former was quite resistant to proteinase while the latter were attacked by proteinase. Despite playing an important role in enzymatic action, the metals did not affect the adsorption of the enzyme on starch. The significance of calcium in natural bacterial α-amylase was thus substantiated clearly.

Recently, Stein and Fischer(1) have reported that calcium contained in α-amylase plays a role in protecting the enzyme from proteolytic attack. Also, the present authors(2) and Fischer et al.(3) from different experiments, have respectively found that bacterial α-amylase inactivated by the removal of calcium is reactivated by the addition of calcium salts. This indicates that the metal contained in the enzyme plays an important role in enzyme action. These observations may be sufficient to give an explanation of the role of calcium which naturally exists in α-amylase protein.

However, in the authors' experiment, it was found that alkaline earth metals other than calcium are also effective for reactivation of the bacterial α-amylase inactivated with ethylenediamine tetraacetate (EDTA), although their effects were less obvious than that of calcium. The media employed for production of bacterial enzyme usually contain magnesium in larger amounts than calcium and, since magnesium is also able to sustain the activity of the amylase, it might be expected that an amylase containing magnesium exists. However, so far, no papers concerning the isolation of magnesium-containing amylase from bacterial culture broth have been published.

The present paper deals with the study on the enzymatic properties, especially on the stability of bacterial α-amylase reactivated by different kinds of alkaline earth metals respectively. The authors' experiment made it clear that calcium amylase is prominent in the stabilty and only calcium amylase shows absolute resistance towards proteinase. This indicates that the stability of bacterial α-amylase strongly depends on the kind of metal bound to the enzyme, providing further evidence for the importance of calcium naturally binding to the enzyme. Also, this fact may point out the reason that magnesium-containing bacterial α-amylase has never been isolated from culture broth. The present paper also describes the effect of alkaline metal ions on the properties of EDTA inactivated amylase.

The mechanism involved in the stabilizing effect of calcium on α-amylase is not yet clarified. But the existence of calcium in the α-amylase preparations successfully crystallized from various sources may serve as a clue for further elucidation of the above mechanism.

1) E. A. Stein and E. H. Fischer, *J. Biol. Chem.*, **232**, 867 (1958).

2) T. Yamamoto and J. Fukumoto, This Bulletin, **23**, 68 (1959).

3) E. H. Fischer, W. B. Sumerwell, J. Junge and E. A. Stein, The 4th International Congress of Biochemistry, Symposium 8-3, 1958.

MATERIALS AND METHODS

1. Bacterial α-amylase and its inactivation with EDTA.

Crystalline bacterial α-amylase[4] obtained from the culture filtrate of *Bac. subtilis* var. *amyloliquefaciens* Fukumoto[5] reported elsewhere was used. However, the treatment of the enzyme in order to remove the contaminating proteinase completely and subsequently to inactivate the enzyme with EDTA was carried out under a somewhat different condition, since the pH in the previous paper was a little too high and sometimes led to poor recovery of the amylase. One gram of the crystalline amylase was suspended in 80 ml of distilled water and dissolved by addition of a minimum amount of N/5 sodium hydroxide solution. After dialyzing the enzyme solution against distilled water for several hours (the pH fell to 6.2), 10 ml of M/5 phosphate buffer, 10 mg of "Potato protease inhibitor"[6] and 0.3 ml of 0.5 M diisopropyl fluorophosphate in isopropanol were added and the solution (pH 6.8) was incubated at 25° for two days. The enzyme solution was then dialyzed against distilled water until the pH fell to 6.1 and, after adding 10 ml of M/20 ammonium-EDTA solution (pH 6.2), the enzyme solution was again incubated for three days at 25°C (pH 5.8, total vol., 124 ml). In this method there sometimes occurred incomplete inactivation of the enzyme. In such cases, however, a further dilution of the enzyme solution several times with M/1000 ammonium-EDTA followed by incubation (pH 5.8) at 30°C for several hours led to the complete inactivation, which was perfectly reversible. In the experiment, the enzyme thus inactivated was used after adequate dilution with a dilute solution of EDTA in order to block metalic contaminants, if any, which might result from glassware and other utensils.

2. Enzyme activity assay.

Enzyme activity was assayed by determining the power of either dextrinization or saccharification of starch.

i) Dextrinization power method. To a mixture of 5 ml of 2% soluble starch, 2 ml of M/20 acetic acid-ammonium acetate buffer and 2 ml of distilled water or salt solutions, one ml of enzyme was added at 30°C (pH of the reaction mixture, 6.0). At varied intervals, 0.5 ml aliquots of the enzyme reaction mixture were taken into 0.5 ml of cold N/1000 iodine solution (2∼4°C) containing N/100 hydrochloric acid and the time required to attain the standard colour (red) of the iodine reaction was measured. Within the range of 3 to 90 min. the time was completely in inverse proportion to the amount of enzyme added. One unit of enzyme activity was defined as the quantity of enzyme that brought the iodine reaction to a standard colour in 60 min., under the above condition. In order to confirm the enzymatic status which should be completely inactive, but reversible, the soluble starch dissolved in ammonium-EDTA solution was used as the substrate (the final concentration of EDTA in the enzyme-reaction mixture, M/1000).

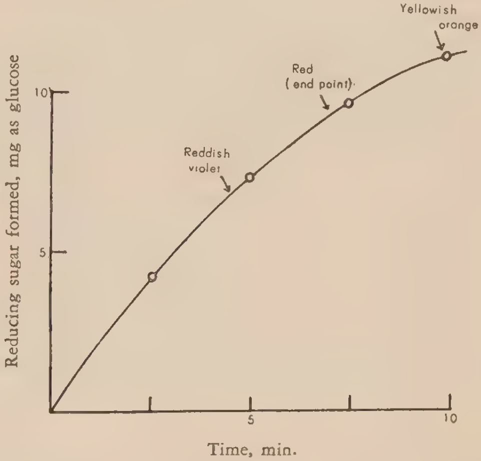

FIG. 1. The Relationship between the Dextrinization and Saccharification Activity of Bacterial-α-Amylase at 30°C

(0.0018 mg of enzyme as N in 10 ml of soluble starch; the total amount of starch, 81.1 mg as glucose after acid hydrolysis).

ii) Saccharification power method. The enzyme reaction for the test was carried out under the same condition as in the dextrinization method. After five min. of reaction, a 3 ml aliquot was taken into a solution of Rochelle salt and sodium hydroxide and the reducing sugar formed was determined by a modification[7] of the Fehling-Lehmann-Schoorl method. In this method, the enzyme activity that formed 1 mg of reducing sugar as glucose in the total reaction mixture under the condition was defined as one unit. The relationship between the dextrinization and saccharification of starch by the enzyme is shown in Fig. 1.

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[4] T. Yamamoto, This Bulletin, **19**, 121 (1955).

[5] J. Fukumoto, *Nippon Nôgei-Kagaku Kaishi* **19**, 487, 634, 689, 789, 853 (1943), **20**, 23, 121, 309 (1944).

[6] K. Matsushima, *Nippon Nôgei-Kagaku Kaishi*, **31**, 38 (1957).

[7] T. Yamamoto, J. Kumada and T. Sawai, This Bulletin, **21**, 185 (1957).

RESULTS

1. Reactivation of the EDTA-inactivated amylase by various alkaline earth metals and the relative activity of the enzyme reactivated.

The EDTA-inactivated enzyme was diluted two hundred times with M/1000 ammonium-EDTA solution and the enzyme was reactivated by combining 1 ml aliquots of the diluted solution with 1 ml of each M/300 acetate solution of various alkaline earth metals. The activity was determined by the saccharification method using soluble starch dissolved in EDTA and supplemented with each of metals salts giving a final concentration of M/500. As shown in Fig. 2, the enzyme without the addition of any metal salt exhibited no enzyme activity at all while the enzyme in combination with metal salts showed enzyme action and their relative activities were calculated as follows: Ca 100, Sr 84, Mg 82, Ba 77 and Be 22. The enzyme added to starch solution without metals was also able to restore its activity to similar degrees when the respective metal salts were subsequently added, even after a four-hour incubation under the condition. This was very convenient to confirm the reversible inactivate state of the enzyme.

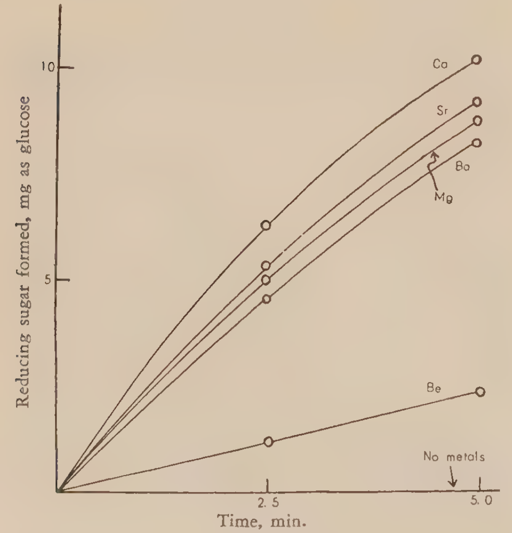

FIG. 2. Relative Activity of Bacterial α-Amylase reactivated with Various Alkaline Earth Metals.

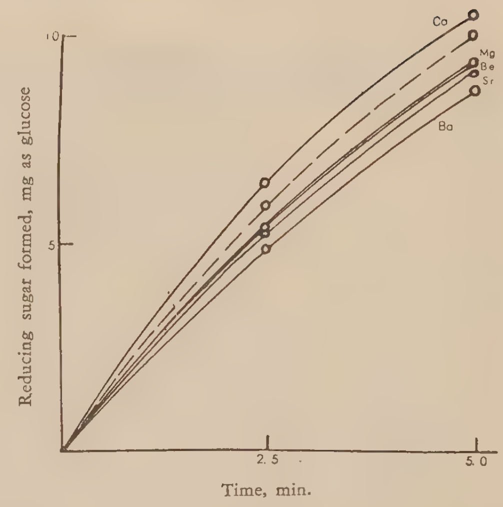

FIG. 3. Enzyme Activity exhibited in the Presence of Calcium by the Bacterial α-Amylase Previously reactivated with Different Alkaline Earth Metals.

The broken line indicates the sugar formation occurred when adding the EDTA-inactivated (metal free) amylase to the calcium supplemented starch.

However, when this test according to this method was carried out at 37°C, as reported previously, the degree of reactivation gradually declined with the time of incubation of the enzyme-substrate mixture, because of instability of the inactive amylase at temperatures exceeding 35°C.

The amylase once reactivated by the addition of any of the metal salts maintained its own specific activity, even in a simultaneous existence of calcium (M/500) in the starch solution. An exception was the beryllium-reactivated amylase which gave a remarkably increased activity in the presence of calcium as shown in Fig. 3.

2. The stability at various pHs of the reactivated amylase.

The EDTA-inactivated enzyme was diluted 30 times with M/1000 ammonium-EDTA and, for reactivation, 10 ml of the enzyme was added to an equal volume of M/300 acetate solution of alkaline earch metals. Then 2 ml aliquots of the solution were combined with 2 ml of M/20 acetic acid-ammonium acetate buffer or Tris-buffer of various pHs and changes in enzyme

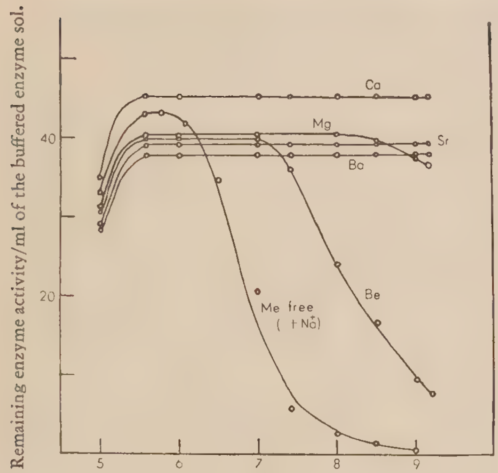

FIG. 4. Comparative pH-Stability of Bacterial α-Amylase reactivated with Various Alkaline Earth Metals (16°C, 4 days).

activity were investigated after the solution was allowed to stand at 16°C for four days. The pH-stability of the EDTA-inactivated amylase was also investigated with or without addition of alkaline metal salts. In the experiment, enzyme activity was assayed by the dextrinization method using calcium-supplemented (M/500) soluble starch buffered with M/20 acetic acid-ammonium acetate. As shown in Fig. 4, the EDTA-inactivated enzyme allowed to stand at the alkaline side over pH 8.0 completely lost its competence of reactivation, regardless of the presence of any alkaline metal salt. The beryllium-reactivated amylase also lost its activity at the alkaline side. On the other hand, the amylase reactivated with other alkaline earth metals showed almost equal stabilities at least up to pH 9.2, irrespective of the metals, only that the magnesium-reactivated amylase slightly lost its activities at the above pH.

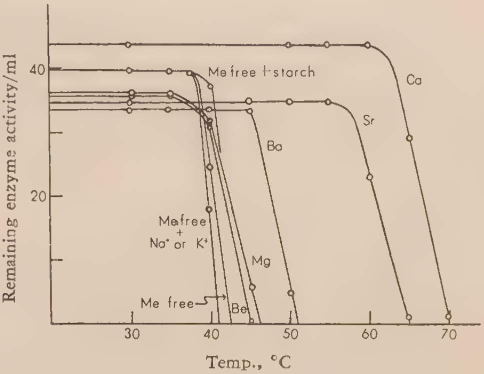

FIG. 5. Heat-stability of Bacterial α-Amylase reactivated with Various Alkaline Earth Metals

(pH 5.8, 15 min.; the inactivated amylase diluted 60 times the original solution with M/1000 NH_4-EDTA, was reactivated by adding to an equal volume of M/300 Me^{++}-acetate and then heated. The inactivated amylase (metal free) was heated in the presence or absence of M/600 Na- or K-acetate or 1% soluble starch dissolved in M/1000 NH_4-EDTA.).

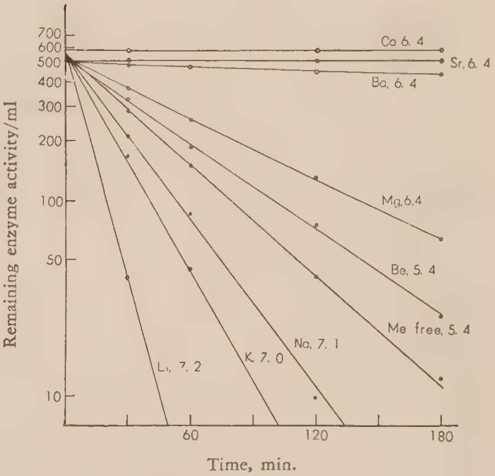

FIG. 6. Changes in Activity of the Reactivated Amylase during the Incubation at 40°C

(the figure also indicates the changes in reactivation competence of the EDTA-inactivated amylase with or without alkaline metal ions under the same condition; the EDTA-inactivated amylase diluted 5 times the original solution with M/100 NH_4-EDTA was added to an equal volume of M/50 Me-acetate and then incubated; assayed the activity in the presence of calcium; the number written in the figure indicate the pH of the enzyme.).

3. The stability of the reactivated amylase at various temperatures.

The enzyme reactivated by the addition of alkaline earth metals in a manner similar to the above experiment was exposed to various temperatures for fifteen min., respectively, followed by immediate cooling to 2°C. The changes in the activity were investigated by the dextrinization method using calcium-supplemented soluble

TABLE I. EFFECT OF *Bac. subtilis* ALKALINE PROTEINASE ON THE REACTIVATED AMYLASE

A. Changes in activity and protein breakdown of the amylase by the proteinase (amylase, 0.037 mg as protein N or 0.0265 mg as tyrosine per ml; the condition for enzyme reactivation, the same as in Fig. 5: 16°C, pH 6.2).

Amylase reactivated with	Be		Mg		Ca		Sr		Ba		None	
Incubation period, hr.	0	1	0	6	0	6	0	6	0	6	0	1
Activity*/ml	149	0.5	146	42	171	170	141	126	126	25	160	0
TCA soluble substance** as tyrosine, γ/ml	1	27	3	21	4	4	4	7	3	22	2	27

* Assayed using calcium-supplemented soluble starch.

** An equal volume of the mixture solution of 0.33 M CCl_3COOH, 1.0 M CH_3COOH and 0.67 M CH_3COONa was added and, after 30 min. at 30°C, the mixture was filtered and a modified Folin-Ciocalteau's method was applied to the filtrate. Tyrosine colour value due to the proteinase was subtracted.

B. Influence of the proteinase on the reactivated amylase in case of low concentrations of the latter (amylase, 0.006 mg as N; the other conditions, the same as A.).

Amylase reactivated with	Be		Mg		Ca		Sr		Ba		None	
pH	6.2	7.2	6.2	7.2	6.2	7.2	6.2	7.2	6.2	7.2	6.2	7.2
Enzyme after 0	23	23	23	23	28	28	22	22	21	21	26	26
0.5 hr.	1	2	—	—	—	—	—	—	—	—	0	0
5.0 〃	0	0	8	12	28	28	18	20	6	9	—	—
17.0 〃	—	—	0	0	27	28	5	14	0	0	—	—

starch. For comparison, a similar experiment was carried out with the inactive amylase with and without addition of alkaline metal salts or soluble starch dissolved together with EDTA. As seen from Fig. 5, the stability to heat of the enzyme significantly varied with the kind of the metal used for reactivation and the degrees of the stability appeared in the following decreasing order: Ca, Sr, Ba, Mg and Be. On the other hand, it will be seen from Fig. 5 that the heat stability of the inactive amylase slightly increased in the presence of starch, but rather decreased by the addition of alkaline metal salts.

Figure 6 shows changes in activity of the reactivated amylases during incubation at 40°C. It indicates that all the enzymes other than calcium- and strontium-reactivated amylase lost their activity during the incubation, following the first order-equation.

4. Resistance of the reactivated amylase to *Bac. subtilis*' alkaline proteinase.

It has been known that natural bacterial α-amylase is not attacked by proteinase unless denatured[8]. Thus, the character has often been used for the determination of degrees of denaturation of the enzyme. However, it was found that this is not true for the bacterial α-amylase reactivated by alkaline earth metals other than calcium. To 3 ml each of the amylase reactivated by different kinds of alkaline earth metal salts respectively, as in the above experiments, 1 ml solution containing 0.1 mg per ml of alkaline proteinase from *Bac. subtilis*[9,10] was added and changes in enzyme activity were investigated at 16°C. In this experiment, the amounts of amylase digested by the proteinase were also investigated by applying a modification[4] of the Folin-Ciocalteau method. As seen in Table I A, only calcium-amylase could maintain its activity through the exposure to the proteinase while this was not observed with other amylases reactivated by other metals. The deactivation proceeded in good consistence with the digestion by the proteinase. However, the strontium-reactivated amylase showed a considerable resistance to the proteinase, the deactivation being significantly slow as compared with the amylase with other metals (Table I B).

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[8] K. Okunuki, B. Hagihara, H. Matsubara and T. Nakayama, *J. Biochem.*, **43**, 453 (1956).

[9] B. Hagihara, *Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ.*, **2**, 35 (1954).

[10] J. Fukumoto, T. Yamamoto and K. Ichikawa, *Nippon Nôgei-Kagaku Kaishi*, **33**, 9 (1959).

5. Adsorption of the reactivated amylase on starch.

Since it is well known that bacterial α-amylase, as well as malt α- and pancreatic amylase, is specifically adsorbed on starch in the presence of certain concentrations of organic solvents or inorganic salts[4)], the experiment was undertaken to ascertain whether the extent of adsorption on starch was affected by the kind of metals used for reactivation. The original solution of the EDTA-inactivated amylase was diluted 10 times with M/400 ammonium-EDTA and, to each 4 ml aliquot of the solution, one ml of M/20 acetate of alkaline earth metals was added. Then, the mixtures were cooled at 4°C and to each 3 ml of cold alcohol was added immediately followed by the addition of 2 ml of a cold 5% soluble starch solution in M/150 ammonium-EDTA (pH 6.0). After five min. the mixture was centrifuged and the supernatant was decanted into another tube while the precipitate was resuspended in distilled water to a final-volume of 10 ml. Then enzyme activity of both fractions was investigated. As seen in Table II, the precipitate fraction from various amylases associated with different metals gave almost equal activities irrespective of the metals when determined with calcium-supplemented starch. Furthermore, the extent of recovery of even the EDTA-inactivated amylase from the precipitate fraction was almost similar to that of the metal-reactivated amylase. The activities in the supernatant fraction, however, considerably differed according to the kind of metal added. This was due to the difference in the stability of those reactivated amylases in alcoholic solution, because the difference in enzyme activity became greater in accordance with the time which the solutions were allowed to stand at room temperature (18°C).

TABLE II. ADSORPTION OF THE REACTIVATED AMYLASE ON STARCH

Amylase reactivated with	Enzyme activity* (total)	
	Supernatant	Precipitate
Be	0	960
Mg	670	1100
Ca	610	1150
Sr	590	1150
Ba	490	1240
None	420	820

* Determined using the calcium-supplemented starch; the amount of amylase used, 2240 as dextrinization activity.

DISCUSSION

In the present experiments, a commercial soluble starch was used as the substrate for investigation of the effect of alkaline earth metals on the EDTA-inactivated amylase. The soluble starch contained nearly 0.1% of calcium which was extraordinarily abundant among the metalic contaminants. However, the chelate formation constant of calcium with EDTA is the greatest among those of alkaline earth metals and there is no possibility that calcium once bound to EDTA is set free in an available form for enzyme by the addition of other alkaline earth metals. Hence, it is not inadequate to use ordinary starch preparation to investigate the effect of individual metals as far as the starch is employed as a solution in EDTA.

In the experiment shown in Fig. 3, it was found that the amylase reactivated with certain alkaline earth metals maintained the specific activity, even in the simultaneous existence of calcium. There remains a possibility, however, that the enzyme activity may become similar to that of calcium-amylase irrespective of the kind of metals, if the amount of calcium is extremely large. Nevertheless, the result shown in Fig. 3 indicates that the metal once taken in the enzyme to support the activity is not so easily substituted by other metals while the enzyme is acting.

The EDTA-inactivated amylase was found to be stable only in the vicinity of pH 6.0. This is probable the true stability and does not seem to be due to incomplete removal of calcium from the enzyme by EDTA at that pH. Otherwise, such result would not occur that the reactivated amylases rather easily deteriorated at the acid side in the presence of bacterial proteinase, with the exception of calcium-amylase (Table I_B).

The fact that the resistance of the amylase to proteinase, as well as its heat-stability, is greatly dependent on the kind of the metal which supports the enzyme activity, seems to show a very important access to the nature of the amylase. Also, this seems to raise a new problem on enzyme nature apart from denaturation that is caused by rupturing the secondary intramolecular bonds of native proteins. Thus, the above fact may lead to the suggestion that the metal-binding site in the enzyme contributes not only to enzyme action, but also to the maintenance of the enzyme protein in an appropriate state. The authors once compared the role of calcium in bacterial α-amylase with the rivet of a fan[11]. It may be said through the present paper that among alkaline earth metals calcium can act best as a rivet for the enzyme. Strontium is comparable to calcium while the others are far less effective. The difference in the effect as the rivet may, of course, be attributed to that in the radius or ionic force of those alkaline earth metals. However, as seen from Table II, it seems that the metal-binding site of the enzyme does not participate in the adsorption of the enzyme on starch.

In the previous papers it was reported that the removal of calcium from bacterial α-amylase caused irreversible inactivation of the enzyme[11], and that neither magnesium nor barium could substitute calcium in the enzyme, as the addition of EDTA-salt of magnesium or barium to the enzyme also resulted in the inactivation of the enzyme[12]. The irreversibility mentioned there has been, however, found through the present experiments to be due to the fact that the previous experiments were carried out under the condition far from the stability of the amylase deprived of calcium or of the amylase in which calcium was substituted with magnesium or barium. Also, in the experiments of formation of bacterial α-amylase, it was found that the amount of amylase formed by bacterial cells greatly decreased by washing the cells with a dilute solution of EDTA and the decrease was reversed by the addition of calcium or strontium, but was not reversed by magnesium. This may also be easily explained from the present findings as follows: the amylase supported by magnesium would once have appeared in the medium but the enzyme had immediately been attacked by the proteinase that was simultaneously secreted by the cells.

The present paper has given further evidence for the role of calcium found in natural bacterial α-amylase, although the mechanism underlying the prominent effect of calcium for stabilization of the enzyme is so far not clarified.

SUMMARY

Bacterial α-amylase was inactivated with ethylenediamine tetraacetate and, after being reactivated by the addition of salts of various alkaline earth metals, respectively, properties of the reactivated amylase were investigated:

1) The enzyme activities of the reactivated amylase in relation to the associated metals decreased in the following order: Ca, Sr, Mg, Ba and Be.

2) The stability of the reactivated amylases at alkaline side was not so significantly different according to the kind of metals, with the exception of beryllium-reactivated amylase which, as well as the EDTA-inactivated amylase, was sensitive to alkaline condition.

3) The stability of the reactivated amylase to heat remarkably varied with the kind of the metals decreasing in the following order: Ca, Sr, Ba, Mg and Be. Though the inactivated amylase was stable at temperatures below 35°C, it easily lost its competence of reactivation at temperatures above 40°C.

4) Only the calcium-amylase exhibited absolute resistance to proteinase. The deterioration by proteinase occurred gradually in strontium-amylase while it proceeded rapidly in the amylases reactivated by other metals.

5) All the reactivated amylases were found to be almost equal in their degree of adsorption on starch, in spite of the remarkable difference in activity and stability.

Acknowledgement. The authors' cordial thanks are due to Emeritus Prof. Izue Yamasaki of Kyushu University and Drs. Edmond H. Fischer and Eric A. Stein of University of Washington for their helpful advice and discussion. Their thanks are also due to Mr. Shigeyoshi Takenaka for his assistance in the analysis.

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[11] T. Yamamoto, This Bulletin, **20**, 188 (1956).

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The Structure of Fungisporin

(Studies on Fungisporin III)

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A molecular weight of 980 was determined for fungisporin by the isothermal distillation method. In view of information published previously[1] this points to a formula (Val, Phe)$_4$ for this compound. Sequence studies on peptide fragments, obtained from fungisporin by partial acid hydrolysis, led to the structural formula cyclo-(Val$_2$·Phe$_2$)$_2$ for this natural cyclic polypeptide. Enzymatic experiments with the peptides obtained, showed that the full formula of fungisporin is most probably cyclo-(D-Val·L-Val·D-Phe·L-Phe·)$_2$.

In the previous paper[1] of this series it was shown that fungisporin, a sublimable component of the spores of some fungi, has the empirical formula $(C_{14}H_{18}N_2O_2)_x$, and is a polypeptide composed of equimolar amounts of valine and phenylalanine residues. The absence of terminal groups was also demonstrated. It was therefore suggested that fungisporin is a cyclopeptide. Supporting evidence for the proposed cyclic structure is to be found in the absence in its I. R. spectrum of 2100–2040 cm^{-1} and 1400 cm^{-1} bands, corresponding to NH_3^+ and $-COO^-$ groups respectively, and in its solubility characteristics. In the present paper the measurement of the molecular weight of fungisporin and the determination of the sequence of its component amino acid residues and their optical configuration is described.

The absence of functional groups in fungisporin made the application of chemical methods for the determination of its molecular weight unfeasible. Also, no suitable solvent could be found for cryoscopic measurements. The use of the isothermal distillation technique[2] using trifluoroacetic acid as the solvent proved very successful, and a molecular weight of 980 was found. From this figure the formula (Phe, Val)$_4$ was derived.

Having established the molecular weight and amino acid composition of the cyclooctapeptide, the next problem was that of its amino acid sequence. Let us consider the possible ways in which four phenylalanine residues (phe) and four valine residues (val) can be joined together to form an eight membered ring. There are ten possible structures which are illustrated in Fig. 1.

The experimental data reported below show that forms I, II, V, VI, VII, VIII and one of the two possible forms of III can be definitely excluded. Evidence of a more indirect nature points to the conclusion that form IV represents the actual structure of fungisporin.

The most conclusive way of determining the amino acid sequence would involve the formation, by partial hydrolysis, of an open octapeptide, its isolation and the determination of its amino acid sequence. The fact, however, that only two different amino acids are present made such a sequence determination too difficult to carry out, although a suitable octapeptide was indeed isolated. Sequence work was there-

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[1] U. Sumiki and K. Miyao, *J. Agr. Chem. Soc. Japan*, **26**, 27 (1952); K. Miyao, This Bulletin, **19**, 86 (1955).

[2] R. Signer, *Ann.*, **478**, 246 (1930).

TABLE I

Peptide S Spot[1]	R_n		$(P/V)_n$		$(P/V)_{n-1}$		Terminal		Structure	
	A[2]	B[3]	A[2]	B[4]	A[2]	B[5]	H_2N-	-COOH	D[6]	E[7]
I	7.65	8	1.03	4:4	0.72	3:4	P	P	$P(P_2, V_4)P$	$PVV(P_2, V_2)P$
II	6.21	6	0.93	3:3	1.38	3:2	V	P	$V(P_2, V_2)P$	VPPVVP
III	5.74	6	0.53	2:4	0.77	2:3	V	V	$V(P_2, V_2)V$	
IV	3.81	4	0.89	2:2	0.45	1:2	P	V	P(P, V)V	
V	3.38	3	0.63	—	0.78	—	P	V	Mixture	
VI	2.32	2 or 3	0.83	—	2.2	—	P, V	P	〃	
VII	2.20	2	0.92	—	0.95	—	P, V	P, V	〃	
VIII	1.79	2	0	0	0	0	V	V	VV	

1) The roman numbers refer to Fig. 2.
2) Experimental values.
3) Nearest whole number.
4) Nearest whole number ratio where $P+V=R_n$.
5) Nearest whole number ratio where $P+V=R_{n-1}$.
6) Structure from amino acid analysis and determinations of terminal residues.
7) From degradation experiments.

TABLE II

Peptide	Hydrolysing Agent	Time of Hydrolysis	Temperature	R_n		$(P/V)_n$	
				A[1]	B[2]	A[1]	B[3]
1	AcOH/HCl	20 min.	120°	3.56	4	0.81	2:2
2	AcOH/HCl	2 hr.	120°	4.28	4	0.79	2:2
3	AcOH/HCl	5 hr.	120°	4.41	4	0.97	2:2
4	1 N NaOEt	20 min.	80°	3.96	4	1.21	2:2
5	1 N NaOEt	1 hr.	100°	4.17	4	1.10	2:2
6	1 N NaOEt	1.5 hr.	120°	3.98	4	0.88	2:2

1) Experimental values. 2) Nearest whole number. 3) Nearest whole number ratio where $P+V=R_n$.

fore based on the determinations in the octapeptide and two hexapeptides obtained.

The best conditions for partial hydrolysis of the cyclopeptide were found to be heating in glacial acetic acid saturated with hydrogen chloride. Satisfactory results were obtained with reaction times of 20 minutes to 5 hours at 80–120°. 1 N sodium ethylate in ethanol was used for the preparation of some tetrapeptides.

Typical chromatograms of partial hydrolyzates are given in Fig. 2. The octapeptide (corresponding to spot I) and the two hexapeptides (spots II and III) were isolated from a hydrolysis mixture by partition chromatography on cellulose powder and purified by rechromatographing on silica gel. Tetrapeptides were isolated by elution from paper chromatograms. Their chain length was established by the dinitrophenylation technique[3]. Dipeptides were isolated by ion-exchange chromatography, using hydrochloric acid of increasing strength as the eluent[4]. The composition and number of re-

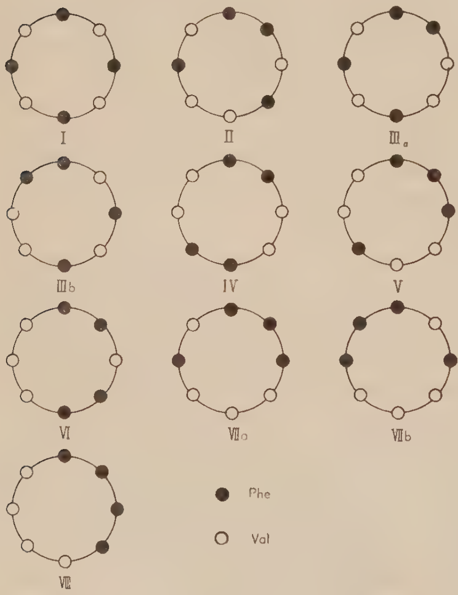

FIG. 1. The Ten Possible Cyclooctapeptide Structures for Cyclo (Phe_4Val_4).

In the diagrams the order of groups is -NH-CHR-CO- when the cycle is traced in a clockwise direction.

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[3] F. A. Isherwood, *Nature*, **174**, 123 (1954).

[4] C. H. W. Hirs, S. Moore and W. H. Stein, *J. Amer. Chem. Soc.*, **76**, 6063 (1954).

TABLE III

No. of peak	Amino Acid Composition	N-terminal Amino Acid	C-terminal Amino Acid	Structure
1	Val_2	Val	Val	Val-Val
2	Val, Phe	Phe	Val	Phe-Val
3	Val, Phe	Val	Phe	Val-Phe
4	Phe_2	Phe	Phe	Phe-Phe

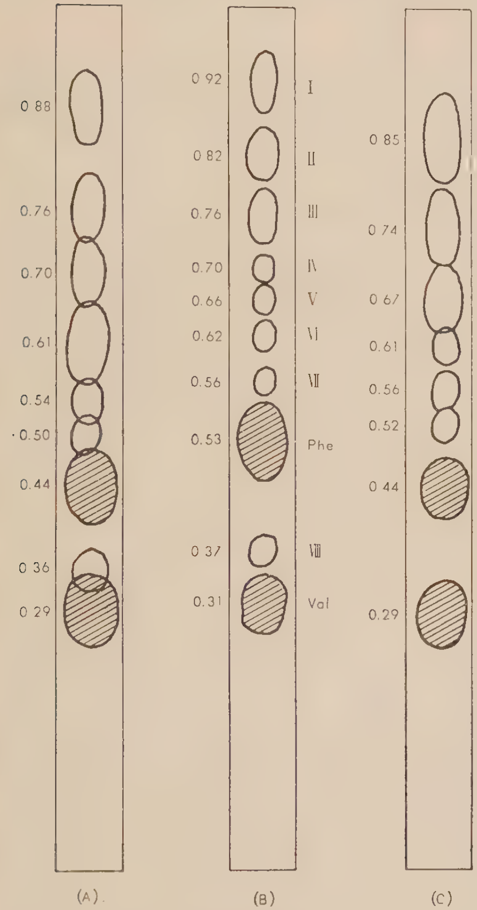

FIG. 2. Paper Chromatograms of Partial Hydrolysates of Fungisporin.

Strip (A) shows a single run chromatogram (partial hydrolysis by acetic acid-hydrogen chloride; developed by isoamylalcohol-pyridine-water). Strip (B) is the same chromatogram after drying and redeveloping with the same solvent system. Strip (C) shows a single run chromatogram (partial hydrolysis by sodium ethylate; developed by the above solvent system). The R_F values are given at the left at the left of each strip; the roman numerales refer to the peptides in the text and the Tables.

sidues of the various spots are summarized in Table I. The composition of additional tetrapeptides isolated is given in Table II. Data on dipeptides are given in Table III.

Information on the structure of the peptides isolated was obtained as follows. All the four dipeptides obtained could be identified unequivocally. This was done by dinitrophenylation followed by hydrolysis. The dinitrophenyl (DNP) amino acids (DNP-valine and DNP-phenylalanine) in the hydrolysate were identified by paper chromatography using aqueous phosphate buffer as developer⁵⁾. The C-terminal amino acids were identified on paper, by developing with isoamyl alcohol-pyridine-water, followed by spraying with ninhydrin. The results can be seen in Table III.

The higher peptides were analyzed for amino acid composition by quantitative paper chromatography of their total hydrolysates. The number of residues in each peptide was estimated from the ratio of total nitrogen to amino nitrogen. In addition both the C-terminal and N-terminal amino acids were identified, the former by the DNP-technique as in the case of the dipeptides, the latter by hydrazinolysis according to Akabori⁶⁾.

In the cases of the octapeptide and one of the hexapeptides, detailed sequence determination was attempted. In addition to the two terminal amino acids, identified as above, the sequence of two more amino acid residues, the second and the third from the N-terminal end, could be established. This was done by the successive application of the thioisocyanate method of Edmann⁷⁾. After the third step in the degradation procedure, however, the reaction mixtures (containing the pentapeptide fragment, derived

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5) A. L. Levy, *Nature*, **174**, 126 (1954).
6) S. Akabori, et al., *Bull. Chem. Soc. Japan*, **25**, 214 (1952).
7) P. Edman, *Acta. Chem. Scand.*, **4**, 283 (1950).

from the octapeptide, or the tripeptide fragment, derived from the hexapeptide) became too contaminated with unspecific degradation products, to yield additional useful information. Chromatograms of the ninhydrin positive components of successive reaction mixtures are given in Fig. 3.

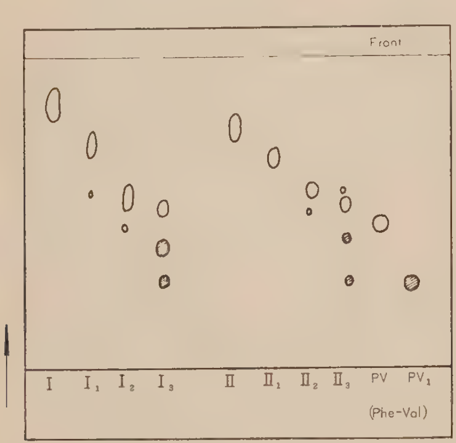


FIG. 3. Paper Chromatogram of Reaction Mixtures obtained by Successive Edmann Degradations of Peptides I and II and of Phenylalanyl-valine.

The indices give the number of degradation steps. Development by isoamyl alcohol-pyridine-water. Spots were revealed by ninhydrin. The shaded spots are the free amino acids.

Essentially all the exact information on amino acid sequence presented here can be deduced from the experiments with the hexapeptide II. Edmann degradation (confirmed by the DNP method) led to the N-terminal sequence Val-Phe-Phe. The C-terminal amino acid residue was found to be Phe. As the total amino acid composition was determined as Phe$_3$Val$_3$ it follows that the total sequence of peptide II is Val-Phe-Phe-Val-Val-Phe. This sequence is compatible only with forms IIIa and IV in Fig. 1. This finding is confirmed by the following additional results. (a) The isolation of the two dipeptides Val-Val and Phe-Phe, excludes form I. (b) Analysis of the hexapeptide III (C-terminal, N-terminal and total amino acid composition) led to the formula Val-(Val$_2$, Phe$_2$)-Val. This peptide contains four valine residues and two phenylalanine residues. It follows that two additional phenylalanine residues must connect the two terminal valine residues of III in the original cyclooctapeptide. This mean that a sequence –Val–Phe–Phe–Val– exists in fungisporin, a fact which excludes forms I, V, VIIa and b, and VIII. (c) An N-terminal sequence of Phe–Val–Val was established for the octapeptide isolated. This sequence together with the C-terminal phenylalanine residue establishes a sequence –Phe–Phe–Val–Val– in fungisporin. This finding excludes forms I, II, IIIb, VIIa and b.

As stated above the two possible structures of fungisporin are form IIIa and form IV. Theoretically it is possible to obtain from form IIIa the two tetrapeptides Phe–Val–Phe–Phe and and Val–Val–Phe–Val. The former has a ratio Phe : Val=3 : 1, the latter a ratio Phe : Val =1 : 3. Now, all the tetrapeptides isolated and analyzed (see Table II) show a ratio of Phe : Val=1 : 1. In addition, the tetrapeptide sequences established by end group analysis and sequence work also gave this ratio. These sequences are : (a) Val–Phe–Phe–Val, Phe–Phe–Val–Val and Phe–Val–Val–Phe in peptide II, (b) a tetrapeptide (Phe$_2$Val$_2$) located between the C and N terminal Val residues in peptide III and the deduced sequence Val–Phe–Phe–Val in this peptide, (c) the established sequence Phe–Phe–Val–Val deduced from the study of the octapeptide. The failure to observe in the products of partial hydrolysis a tetrapeptide sequence of a Phe : Val ratio different from 1 : 1 strongly points to form IV as the actual structure of fungisporin. Additional support for this structure will be found in the following section dealing with the steric configurations of the dipeptides isolated.

The complete analysis of the structure of fungisporin must also deal with the optical configurations of the amino acid constituents. It was found that fungisporin shows no optical rotation in trifluoroacetic acid solution. Moreover, it was observed that both the phenylalanine and the valine, isolated by partition chromato-

TABLE IV

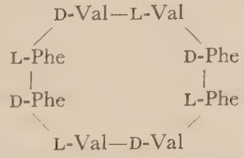
Peptide No.	Structure	A[1]	B[2]	Configuration
1	Val-Val	1 mole L-Val	0.0	D-Val-L-Val
2	Phe-Val	1 mole L-Phe	1.0	L-Phe-D-Val
3	Val-Phe	1 mole L-Val	—	L-Val-D-Phe
4	Phe-Phe	1 mole L-Phe	—	(D, L-Phe$_2$)

1) Product obtained from 1 mole of peptide after total hydrolysis followed by treatment with D-amino acid oxidase.
2) Moles of amino acid oxidized by D-amino acid oxidase after Edmann degradation of 1 mole of peptide.

graphy on cellulose powder columns from a total hydrolyzate, were optically inactive. (The hydrolysis conditions used caused no recemisation when applied to authentic samples of L-phenylalanine and L-valine). These findings imply that the fungisporin molecule contains two residues each of L-phenylalanine, D-phenylalanine L-valine and D-valine.

In order to determine the location of the D and L residues in the original material the following experiments were carried out with the four dipeptides recorded in Table III. Each dipeptide was totally hydrolyzed, the resulting hydrolyzates were treated with hog kidney D-amino acid oxidase and the reaction mixtures were subjected to quantitative paper chromatography. In addition, peptides 1 and 2 were degraded by the Edmann method and the reaction mixtures analysed manometrically using D-amino acid oxidase. The results of these experiments are summarized in Table IV.

It seems likely that the four dipeptides isolated by column chromatography originate each from different parts of the fungisporin molecule as a result of a more or less random partial hydrolysis. Making this assumption, there is only one way in which the optical isomers of the two amino acids can be arranged in the structural formula derived above. The complete formula of fungisporin seems therefore to be:



The alternating arrangement of D and L amino acids in the cyclopeptide is further supported by the observation that peptides I, II, III, as well as their DNP derivatives and the four dipeptides were completely resistant toward the action of crystalline pepsin, pancreatic carboxypeptidase, the proteolytic enzyme of Black Aspergillus[8] and of a Streptomyces species[9].

EXPERIMENTAL

I. Determination of molecular weight by the isothermal distillation method.

All measurements were carried out using trifluoroacetic acid as solvent. Preliminary experiments by the Barger-Akiya[10] method were not well reproducable and gave molecular weights of 750~1200.

The procedure adopted finally was based on the method of Signer[2]. The original apparatus was modified so as to permit the use of micro samples. Employing small volumes considerably reduced the time necessary for equilibration, thus obviating decomposition during

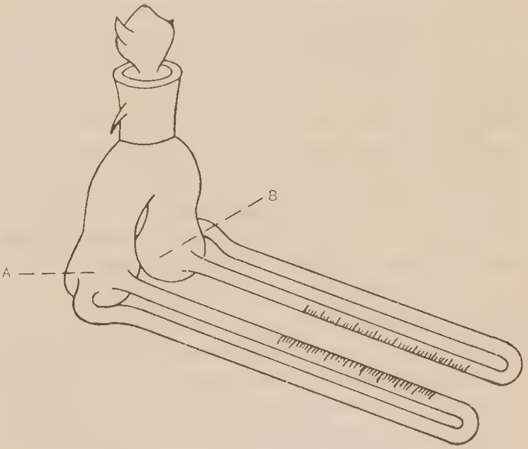


FIG. 4. Apparatus for the Determination of Molecular Weight by Isothermal Distillation.

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[8] M. Nomoto, Lecture delivered in July, 1956, at the meeting of "Symposium on Enzymology" (Tokyo, Japan).
[9] F. Yoshida, This Bulletin, **22**, 32 (1958).
[10] T. Akiya, *J. Pharm. Soc. Japan*, **57**, 567 (1937).

measurement. Fig. 4 shows the instrument as employed. The two bulbs have a diameter of 1.5 cm, the measuring capillaries were of 1.6 mm inner diameter, corresponding to approximately 10 cm of column height per 0.1 ml liquid. They were graduated in 0.001 ml divisions, and calibrated by means of mercury. The U-form of the capillaries greatly facilitates the transfer of the small volumes during measurement.

About 5 to 6 mg of the sample and 1 to 2 mg of a standard substance (either azobenzene or diketopiperazine) were weighed into bulbs A and B respectively by means of a microbalance. Approximately 0.2 ml of the solvent was introduced into each bulb, the instrument was closed by means of the glass stopper, slightly lubricated with silicone grease, sealed off with melted paraffin and the seal protected by means of a piece of plastic film held in place by a rubber band. The apparatus was completely immersed in a constant temperature water bath (35°C) and lightly shaken. After complete solution of both sample and standard the initial reading was taken. Additional measurements were made at two hour intervals until equilibrium was reached. The molecular weight, M, of the substance was calculated from the formula $M=M'\times S\times V'/S'\times V$ where M' is the molecular weight of the standard substance employed S and S' the weights of sample and standard respectively and V and V' the final volumes of the solutions containing the sample and the standard respectively. The average value obtained for fungisporin from 16 determinations was $M=980\pm50$.

II. Amino acid sequence

A) Partial hydrolysis. Partial acid hydrolyses was carried out in glacial acetic acid saturated at 0° with hydrogen chloride gas. Suspensions of 10 to 100 mg quantities of fungisporin in 1 to 10 ml of the hydrolysing agent contained in sealed test tubes were heated at 80 to 120° in a constant temperature bath for the required time. The reaction mixture was then filtered, taken to dryness and the residue was dissolved in the appropriate solvent used for separation.

Partial alkaline hydrolysis was carried out in 1 N sodium ethylate (1 ml per mg substance) in sealed tubes at 80 to 120° for the required time. The reaction products were treated first with water, then with acetic acid and taken to dryness.

B) Paper chromatography of partial hydrolysate.

(1) All chromatographic separations on paper were carried out on Whatman No. 1 filter paper using the ascending technique. The developing solvent employed for peptides was pyridine-isoamyl alcohol-water (35 : 35 : 30 by volume), whereas for the separation of amino acids butanol-acetic acid-water (4 : 1 : 5 by volume) was used. Spots were revealed either with ninhydrin (0.2% in butanol) or by Reindel's[11] method.

In the case of complex mixtures, improved separations on paper could be obtained by double development. After the first development the paper was dried at room temperature and redeveloped with the same solvent before spraying (see Fig. 2).

(2) Preparative isolation of peptides as their dinitrophenyl derivatives: The products of partial hydrolysis obtained as described above were dissolved in glacial acetic acid (to a concentration of about 2%) and applied to several sheets of paper (20×40 cm), about 5 mg per sheet, in the form of a line near the base. Double development, 10～15 hours for the first run, 20 hours for the second run, was employed. Two guide strips were cut from the edges of each sheet, developed by ninhydrin, and used to locate the peptide zones. The main parts of the sheets were sprayed with a freshly prepared DNP reagent according to Isherwood[3] (60 mg of 2,4-dinitrofluorobenzene, 33 ml ethanol and 17 ml 0.2 M borate buffer, pH 8.5), and kept in a closed vessel for 5 hrs. at 50°C. The yellow zones formed were cut out, with the help of the guide strips, wetted with 1 N HCl and extracted with ethyl acetate. Corresponding extracts were combined and evaporated to dryness in a current of air. The residues were heated at 50°C and 0.01 mmHg in a lyophilyzing apparatus equipped with a cold trap (−50°C) in order to remove dinitrophenol. The residues thus obtained were dissolved in ethyl acetate, brought to an appropriate volume and the solutions (to be called "sol. A" in the following) used for the quantitative determinations described below.

(3) Analysis of peptides and their DNP-derivatives:

(3a) Determination of total amino acid nitrogen: An aliquot of "sol. A" (containing 0.5～15 μg amino acid N) was pipetted into a test tube (1.5×16 cm). Digestion and colorimetric ammonia estimation was carried out according to Boissonnas[12]. This determination gives the molar concentration of amino acid residues.

(3b) Determination of number of residues per peptide: The optical density of an aliquot of "sol. A" was measured at 350 mμ. Using a value of 14500 for the molar extinction of the N-DNP group, the molar concentration of the solution was calculated. Dividing the molar concentration of residues (determined as in

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[11] F. Reidel and W. Hoppe, *Ber.*, **87**, 1103 (1954).

[12] R. A. Boissonnas and C. H. Haselbach, *Helv. Chim. Acta.*, **36**, 576 (1953).

(3a) by the molar concentration of the peptide gave the number of amino acid residues in the peptide (Table I, R_n, column A).

(3c) Qualitative determination of the C-terminal residue: A part of "sol. A" was evaporated to dryness in a test tube. After the addition of 0.5 ml of anhydrous hydrazine the tube was sealed and heated at 130°C for 3 hours[6]. Excess hydrazine was removed in vacuo over H_2SO_4 and the C-terminal amino acid was identified by paper chromatography. Whenever the amino acid hydrazides present interfered with this determination, the amino acid was isolated by paper electrophoresis prior to chromatography.

(3d) Total hydrolysis of DNP-peptides: A part of "sol. A" was evaporated to dryness in a test tube. After addition of 1 ml of the hydrolysing reagent (conc. HCl-acetic acid, saturated with HCl, 1 : 1 by volume) the tube was sealed and heated at 130°C for 40 hours. The hydrolysate was brought to dryness and the residue exhaustively extracted with ethyl acetate ("sol. B"). The residue was dissolved in 0.1 ml 50% aqueous ethanol ("sol.C").

(3e) Qualitative determination of the N-terminal residue: The "sol. B" was evaporated to dryness, dissolved in one drop of ethyl acetate and chromatographed directly on paper using 1.5M phosphate buffer, pH 6.5, as developer[5]. The DNP-amino acids appeared as yellow spots.

(3f) Determination of the ratio $(\mathrm{Phe/Val})_{n-1}$: Appropriate quantities of "sol. C" were applied to filter paper and chromatographed using butanol-acetic acid for development. The molar ratio of the phenylalanine and valine spots was determined using the method for Schwerdlfeger[13]. This gave the Phe/Val ratio for the peptide minus its N-terminal amino acid residue ($(P/V)_{n-1}$, column A in Table I). Combining this value with the information obtained from (3e) (N-terminal residue) and (3b) (number of residues in the peptide) the composition of the peptide was evaluated (column D in Table I).

(3g) Determination of the ratio $(\mathrm{Phe/Val})_n$: Peptides analyzed for this value (see Tables I, II) were totally hydrolysed as in (3d) and the ratio phenylalanine to valine was determined as in (3f).

C) Isolation of larger peptides by column partition chromatography, and their degradation.

(1) Isolation: A column (2 cm × 50 cm) was prepared from Whatman cellulose powder, equilibrated with the solvent (pyridine-isoamylalcohol-water 35 : 35 : 30 by volume). 100 mg of a partial hydrolyzate (see II, A), dissolved in the above solvent was applied to the column and developed at a flow rate of 5 ml/hr. Fractions of 2ml each were collected automatically. Bands were located by ninhydrin tests on a few drops of the effluent. In the region of a band every other fraction was tested by paper chromatography (see II, B). First peptide I emerged, the initial fractions being chromatographically pure. The following fractions contained a mixture of peptides I and II. (This mixture as well as subsequent impure bands were rechromatographed on silica as described below.) Then came a band consisting of peptide II in a pure state, followed by peptide III, contaminated by a trace of peptide IV. The following additional bands were observed but were not used for isolation purposes: III+IV+V; IV+V+VI+VII; V+VI+VII+Phe; Phenylalanine; VIII+Val; valine.

For rechromatography the fractions comprising each impure band were combined, evaporated to dryness and dissolved in the solvent ethylacetate-acetic acid-butanol (90 : 5 : 5 by volume), The solution were applied to columns prepared as follows: 60g Malinkrodt A.R. silicic acid was mixed with 40 ml of 60% (by volume) aqueous acetic acid, and the nearly dry powder thus obtained was suspended in benzene. The suspension was poured into chromatographic tubes to form 2.5 cm × 40 cm columns. On development, carried out as above, both mixtures (I+II and III+trace of IV) could be resolved into their components. The combined yields of pure peptides from 100 mg hydrolyzate were:

Peptide I: 3.2 mg; Peptide II: 3.6 mg; Peptide III: 5.7 mg.

(2) Degradation: Peptides I and II were subjected to the Edmann degradation[7], using a modified procedure[14, 15].

Reaction of the peptides with phenylisothiocyanate was carried out in pyridine[14]. Excess reagent was removed by evaporation in a desiccator rather than by washing as the phenylthiocarbamyl peptides were considerably soluble in benzene. Cleavage and cyclisation to the thiohydantoins of the terminal amino acid residues was accomplished by means of acetic acid saturated with hydrogen chloride[15]. The thiohydantoins formed were extracted from the residual peptide with benzene, freed from most of the accompanying diphenylthiourea by extraction with hexane and identified by paper chromatography[16]. The composition of the developing solvent (pyridine-hexane) was adjusted so as to optimal separation between the thiohydantoin and the contaminating diphenylthiourea.

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[13] E. Schwerdlfeger, *Angew. Chem.*, **70**, 188 (1958).

[14] W. A. Landman, *J. Amer. Chem. Soc.*, **76**, 3638 (1954).

[15] H. Frankel-Conrat and J. I. Harris, *J. Amer. Chem. Soc.*, **76**, 6058 (1954).

The residual peptides (I_1 and II_1 in Fig. 3) were examined by paper chromatography and then subjected to further degradation. As can be seen from Fig. 3, the third residual peptide (I_3 and II_3) were, in both cases, too contaminated to allow an additional degradation step.

The N-terminal sequences thus estabished for peptides I and II were Phe-Val-Val and Val-Phe-Phe respectively.

D) Isolation and identification of dipeptides.

(1) Isolation by ion exchange columns: 100 mg of the partial hydrolyzate (see II, A) was applied to columns of Dowex 50 and developed by means of aqueous hydrochloric acid of gradually increasing strength (1 N to 4 N) according to Hirs, Moore and Stein[4]. Six well separated bands appeared in the effluent. The first two were phenylalanine and valine. The following four were (in this order, see Table III) Val-Val, Phe-Val, Val-Phe, Phe-Phe, identified as described below. Each band was found to be homogeneous by paper chromatography, and yielded one of the above peptides as its hydrochloride on evaporation. The quantities isolated were 7.6, 4.3, 3.2 and 1.2 mg respectively. Additional bands appeared on further elution but these were partially overlapping and found to be impure by paper chromatography.

(2) Identification: Each peptide fraction was dinitrophenylated according to Sanger[17], and the DNP-peptides obtained were hydrolysed as in IIB, 3d. The DNP-amino acid (N-terminal) was identified on paper (as in IIB, 3c). The C-terminal amino acid was identified by paper chromatography using butanol-acetic acid as developer and ninhydrin to locate the spot.

In one case (Phe-Val) the Edmann[7] procedure was also applied. The result checked with that of the DNP-method.

E) Optical rotation of fungisporin. Fungisporin was found to have no measurable optical rotation when examinated in trifluoroacetic acid solution in a concentration of 10 mg/ml.

F) Optical configuration of amino acids in total hydrolyzate.

(1) Hydrolysis and isolation of amino acids: Fungisporin (23.6 mg) was hydrolysed under the conditions described in IIB, 3d. The dried hydrolyzate was dissolved in butanol-acetic acid-water (4 : 1 : 5 by volume) and chromatographed on a cellulose powder column prepared with this solvent as described in IIC, 1. From the effluent phenyl alanine and valine were isolated in a crystalline form and checked for purity by paper chromatography.

(2) Action of D-amino acid oxidase on the isolated amino acids: Solutions containing about 0.3 mg of the amino acid were analysed manometrically for their D-amino acid content in a Warburg apparatus. The enzyme preparation used was aceton dry hog kidney powder[18]. A sample of 234 μg of phenylalanine isolated as above contained 118 μg (50.5 %) of D-phenylalanine, whereas 308 μg of valine were found to contain 155 μg (50.4 %) of D-valine.

When authentic L-phenylalanine was subjected to the above hydrolysis conditions, it was found to contain no measurable quantity of D-phenylalanine.

G) Optical configuration of dipeptides. The dipeptides isolated as in IID, 1 were totally hydrolysed and the hydrolyzates were incubated with the D-amino-acid oxidase preparation[18] at 25° for 2hr. in 0.2 M pyrophosphate buffer (pH 8.3). The reaction mixtures were quantitatively analysed by paper chromatography as in IIB, 3f. The results obtained are summarized in Table IV. The C-terminal value of valyl-valine and of phenylalanyl-valine, obtained by the Edmann degradation of each of these peptides (see IIC, 2), was analysed manometrically for D-valine content as described above. Results are also given in Table IV.

Acknowledgements. The author is very grateful to Prof. Y. Sumiki for his guidance and encouragement. He is also indebted to Mr. S. Mizushima, Tokyo University, for his help in the experiments with D-amino acid oxidase, to Prof. I. Noguchi, Kanazawa University, for his gift of synthetic peptides, to Prof. T. Ando, Tokyo University, to Dr. F. Yoshida, Noda Institute for Scientific Research, and to Dr. M. Nomoto, the Institute of Physical and Chemical Research, for their kind supplies of enzyme preparations.

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16) J. Sjoquist, *Acta. Chem. Scand.*, **7**, 447 (1953).

17) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463, 481 (1951).

18) This preparation was kindly given by Dr. Mizushima, Dept. of Agr. Chem., Univ. of Tokyo.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 1, p. 31~36, 1960]

Studies on Amino Acids. XIII.

A Survey of the ε-Lysine Acylase Activity in Microorganisms

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Screening experiments for ε-lysine acylase activity in microorganisms have been carried out. Namely, a number of bacteria, streptomyces, molds and yeasts were cultured on the medium containing ε-acyllysines, and ε-lysine acylase activity was estimated by liberated amino acid detected with paper chromatography of the cultural broth.

As a result, the occurrence of ε-lysine acylase activity was observed in molds, especially in *Aspergillus oryzae*. In other organisms such as bacteria, streptomyces and yeasts, the activity was scarcely found under the conditions employed by the authors.

By the same procedure employing acetylmethionine and ε-benzoyl-α-acetyllysine, acylase activity, that is, hydrolytic activity toward α-N-acyl amino acids, of the microorganisms was investigated.

Greenstein and his co-workers[1,2] reported for the first time on ε-lysine acylase, which hydrolyses ε-N-acyl lysine, in rat tissues. Kameda and his co-workers[3] also studied ε-lysine acylase activity in a bacterium of pseudomonas species isolated from soil.

In the previous papers, the authors have reported that acylase activities, hydrolytic activities toward α-N-acyl amino acids, occur not only in animal tissues but also in molds[4,5], bacteria[6], yeast[7] and plants[8]. The present authors also utilized fungal acylase activity for the enzymatic resolution of racemic amino acids[5,9~11]. As for the optical resolution of DL-lysine, the authors established a very advantageous method consisting of asymmetric hydrolysis of ε-benzoyl-α-acetyl-DL-lysine with mold acylase followed by the chemical hydrolysis of the ε-benzoyl-L-lysine obtained[10,11].

Although, if a proper enzyme source showing higher ε-lysine acylase activity is found, L-lysine is also readily obtained by the asymmetric hydrolysis of ε-benzoyl-DL-lysine. Since the distribution of ε-lysine acylase except that above mentioned is entirely unknown, the authors investigated the occurence of the activity in microorganisms for the purpose of finding out advantageous enzyme sources. Besides the experiments conducted to make a survey of ε-lysine acylase, a screening test for α-acylase activity (a general acylase was designated so as to distinguish ε-lysine acylase) was simultaneously carried out in order to serve for reference.

MATERIALS AND METHODS

Compounds Used—ε-Benzoyl-L-lysine was prepared by the asymmetric hydrolysis of ε-benzoyl-α-acetyl-DL-lysine with mold acylase according to the method des-

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[1] W.K. Paik, L. Bloch-Frankenthal, S.M. Birubaum, M. Winitz and J.P. Greenstein, *Federation Proc.*, **16**, 230 (1957).

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[3] Y. Kameda, E. Toyoura, Y. Kimura and K. Matsui, *Chem. Pharm. Bull. Japan*, **6**, 394 (1958).

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[8] I. Chibata and T. Tosa, This Bulletin, **23**, 370 (1959).

[9] S. Yamada, I. Chibata and S. Yamada, *J. Pharm. Soc. Japan*, **75**, 113 (1955).

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[11] I. Chibata, A. Watanabe and S. Yamada, This Bulletin, **21**, 291, 296 (1957).

cribed in the previous paper[11]. ε-Acetyl-L-lysine was prepared by the reaction of L-lysine-copper complex salt with acetic anhydride according to the method given by Neuberger and Sanger[12]. The preparation of ε-benzoyl-α-acetyl-L-lysine and acetyl-L-methionine were the same as previously described[5].

Organisms—About 110 strains of bacteria, streptomyces, molds and yeasts were used in the screening tests. These organisms were supplied from the Institute of Applied Microbiology, Tokyo University, the Department of Fermentation Technology, Osaka University, and the Research Institute for Microbiological Diseases, Osaka University.

Media—Composition of the media suitable for growth of test organisms, containing less ninhydrin colored substance was examined, and the screening media described in the Table I were employed. In the media for the test of α-acylase, ε-benzoyl-α-acetyl-L-lysine and acetyl-L-methionine were added instead of acyllysines.

TABLE I. COMPOSITION OF THE MEDIA FOR THE SCREENING OF ε-LYSINE ACYLASE ACTIVITY IN MICROORGANISMS

Bacteria and Streptomyces		Molds and Yeasts	
Glucose	3.0 %	Malt extract	3.5° Bg.
Meat extract	0.1 %	Glucose	3.0 %
Urea	0.1 %	$(NH_4)_2SO_4$	0.2 %
Peptone	0.05 %	$CaCO_3$	2.0 %
NH_4Cl	0.1 %	ε-Benzoyl-L-Lysine*	0.05 M
K_2HPO_4	0.1 %	ε-Acetyl-L-Lysine*	0.05 M
$MgSO_4 \cdot 7H_2O$	0.05 M	pH	7.5
$CaCO_3$	3.0 %		
ε-Benzoyl-L-Lysine*	0.05 M		
ε-Acetyl-L-Lysine*	0.05 M		
pH	7.2		

* In the medium for the screening of α-acylase, ε-benzoyl-α-acetyl-L-lysine 0.05 M and acetyl-L-methionine 0.05 M are employed instead of ε-benzoyl-L-lysine and ε-acetyl-L-lysine.

Assay of Enzyme Activity—Slant culture for inoculum of the tested organism was carried out on a peptone meat-extract medium for bacteria and streptomyces and on malt extract medium for molds and yeasts.

The respective media listed in Table I were distributed in amounts of 3 ml to test tubes and sterilized. After inoculation with a loopful of the organism, incubation was carried out at 27° or 37° for an appropriate period. Routine identification and a rough quantitative estimation of liberated amino acid was made by paper chromatography of the broth. Paper chromatopraphy was carried out with the ascending method using Tōyō filter paper No. 51 and butanol : acetic acid : water = 4 : 1 : 1 as the solvent. After development for about 20 hours, the paper chromatogram was treated with a 0.2% ninhydrin aqueous butanol solution.

Estimation of ε-lysine acylase activity was made by detection of the spot of lysine and decrease in the spots of ε-acyllysine. On the other hand, α-acylase activity was measured by the appearance of the spots of methionine or ε-benzoyllysine.

RESULTS

Investigation of Bacteria—Twenty-six strains of bacteria were tested in the screening experiments for ε-lysine acylase and α-acylase activities.

As shown in Table II, neither the distinct appearance of lysine spot nor a decrease in the spots of ε-acyllysines were detected on the paper chromatograms. Namely, ε-lysine acylase activity was not found in the bacteria tested under the conditions employed.

In the screening experiments for α-acylase activity, although no spot of ε-benzoyllysine was detected, acetylmethionine was hydrolyzed and a methionine spot was found in the broth of almost all bacteria tested.

Investigation of Streptomyces—Several strains of streptomyces and others were investigated for their acylase activities.

As shown in Table III, ε-lysine acylase activity of streptomyces is scarcely observed under the conditions employed.

On the other hand, the organisms showed higher α-acylase activity, especially toward acetylmethionine.

Investigation of Molds—About 60 strains of molds were tested, and the results obtained are shown in Table IV.

As shown in the Table, the appearance of a lysine spot and simultaneous decreasing of spots of ε-acyllysines were observed in number of the tested molds, especially in the strains of *Aspergillus oryzae*. Among the tested organisms, *Aspergillus oryzae* No. 10, 51 and SZ strains

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12) A. Neuberger and F. Sanger, *Biochem. J.*, **37**, 515 (1943).

TABLE II. ACYLASE ACTIVITIES IN BACTERIA

		ε-Lysine Acylase Activity								α-Acylase Activity					
				Detected Amino Acids**								Detected Amino Acids**			
Organisms	Incubation	Growth* (days)		Lysine (days)		ε-Acetyl-lysine (days)		ε-Benzoyl-lysine (days)		Growth* (days)		Methionine (days)		ε-Benzoyl-lysine (days)	
	°C	2	5	2	5	2	5	2	5	2	5	2	5	2	5
Pseudomonas aeroginosa HO	37	++	++	−	−	++++	++++	+++	+++	++	++	+	+++	−	−
〃 MK	〃	++	++	−	−	++++	++++	+++	+++	++	++	++	+++	−	−
〃 MO	〃	++	++	−	−	++++	++++	+++	+++	+	++	+	+++	·	−
〃 NK	〃	++	++	−	−	++++	++++	+++	+++	++	++	+	+++	·	−
Pseudomonas fluorescens Migula	27	++	++	−	−	++++	++++	+++	+++	++	++	++	++	−	±
〃 6009–2	〃	+	++	−	−	++++	++++	+++	+++	+	+	+	++	−	−
〃 A13–12	〃	+	++	−	−	++++	++++	+++	+++	±	+	−	±	−	−
Pseudomonas miyamizu	〃	+	+	−	−	++++	++++	+++	+++	++	++	++	++	−	−
Pseudomonas myxogenes	〃	+	++	−	−	++++	++++	+++	+++	+	+	++	++	−	−
Pseudomonas nishinomiyaensis	〃	±	+	−	−	++++	++++	+++	+++	±	++	−	±	−	−
Micrococcus flavus	〃	+	+	−	−	++++	++++	+++	+++	+	+	−	+	−	−
Micrococcus candidus	〃	+	+	−	±	++++	++++	+++	+++	+	+	−	+	−	−
Micrococcus pyogenes var. *aureus*	37	+	+	−	−	++++	++++	+++	+++	±	+	±	+	−	−
Achromobacter butyri	27	+	+	−	−	++++	++++	+++	+++	±	±	−	++	−	−
Achromobacter candicans	〃	±	+	−	−	++++	++++	+++	+++	±	±	−	−	−	−
Esherichia coli K–12	37	+	+	−	−	++++	++++	+++	+++	+	+	++	++	−	−
〃 K–12 Ki–1023	〃	+	+	−	−	++++	++++	+++	+++	+	+	++	++	−	−
〃 K–12 Ki–1101	〃	+	+	−	−	++++	++++	+++	+++	+	+	+++	+++	−	−
〃 K–12 Ki–1102	〃	+	+	−	−	++++	++++	+++	+++	+	+	++	+++	−	−
〃 Castellaniet Chalmers	〃	+	+	−	−	++++	++++	+++	+++	+	+	++	++	−	−
Aerobacter aerogenes B–1	〃	+	+	−	−	++++	++++	+++	+++	+	+	+++	++	−	−
〃 Beijerinek	〃	+	+	−	±	++++	++++	+++	+++	±	+	−	±	−	−
Serratia marcescens	27	++	++	−	−	++++	++++	+++	+++	++	++	++	+++	−	−
Proteus vulgaris	〃	+	+	−	−	++++	++++	+++	+++	+	+	++	++	−	−
Bacterium megatherium	〃	±	+	−	−	++++	++++	+++	+++	+	+	−	++	−	−
Bacillus subtilis	37	+	+	−	±	++++	++++	+++	+++	+	++	++	++++	−	±

* + Indicates the relative amounts of growth.

** + Indicates the relative intensity of the spots on the paper chromatogram, ++++ corresponds approximately the intensity of the spot of 0.05 M amino acid.

TABLE III. ACYLASE ACTIVITIES IN STREPTOMYCES

		ε-Lysine Acylase Activity								α-Acylase Activity					
				Detected Amino Acids								Detected Amino Acids			
Organisms	Incubation	Growth (days)		Lysine (days)		ε-Acetyl-lysine (days)		ε-Benzoyl-lysine (days)		Growth (days)		Methionine (days)		ε-Benzoyl-lysine (days)	
	°C	4	9	4	9	4	9	4	9	4	9	4	9	4	9
Streptomyces lavendulae ATCC 8664	37	+	++	±	+	++++	++++	+++	+	+	++	++	++++	·	−
Streptomyces griseus 〃 3463	〃	++	++	−	−	++++	++++	+++	+++	++	++	++++	++++	−	−
Streptomyces rubrireticuli 〃 3631	〃	+	++	−	±	++++	++++	+++	+++	+	++	+++	+++	+++	++
Nocardia gardneri 〃 9604	27	+	++	±	±	++++	++++	+++	+++	+	++	−	−	−	−
Mycobacterium 〃 607	37	++	++	−	−	++++	++++	+++	+++	++	++	+	+++	−	−

showed highest ε-lysine activity. Speaking of the ε-acyllysine used, decrease or disappearance of the spot was observed more often in the case of ε-benzoyllysine than that of ε-acetyllsine.

TABLE IV. ACYLASE ACTIVITIES IN MOLDS

Organisms	Incubation	ε-Lysine Acylase Activity								α-Acylase Activity					
		Growth		Detected Amino Acids						Growth		Detected Amino Acids			
				Lysine		ε-Acetyl-lysine		ε-Benzoyl-lysine				Methionine		ε-Benzoyl-lysine	
		(days)		(days)		(days)		(days)		(days)		(days)		(days)	
	°C	4	9	4	9	4	9	4	9	4	9	4	9	4	9
Aspergillus melleus	27	++	++	−	−	++++	++	+++	+++	++	++	+	−	−	++
Aspergillus parasitics	〃	++	++	−	±	++++	±	++	++	++	++	−	−	+	+++
Aspergillus clavatus	〃	++	++	−	−	++++	++++	+++	+++	++	++	−	+	−	±
Aspergillus flavus	〃	++	++	+	+++	++++	+++	+++	+	++	++	−	−	±	+++
Aspergillus nidulans	〃	++	++	−	−	++++	+++	+++	+++	++	++	−	−	−	+
Aspergillus sydowii	〃	++	++	−	−	++++	−	+++	+++	++	++	−	−	−	−
Aspergillus fumigatus	〃	++	++	−	−	++++	++++	+++	+	++	++	−	−	−	++
Aspergillus tamari No 53	〃	++	++	−	+	++++	+++	+++	+++	++	++	−	−	−	+++
〃 A 119	〃	++	++	−	±	++++	+++	+++	+++	++	++	−	−	−	++
Aspergillus wentii	〃	++	++	−	+	+++	+++	++	++	++	++	−	+++	++	+++
Aspergillus ochracens	〃	++	++	±	−	++++	++++	+++	++	++	++	−	−	−	+++
Aspergillus terreus	〃	++	++	−	−	++++	++++	+++	+++	++	++	−	−	−	−
Aspergillus oryzae No 1	〃	++	++	+	+++	+++	++	++	−	++	++	−	−	−	++
〃 5	〃	++	++	−	±	++++	+++	+++	++	++	++	−	−	−	+++
〃 7	〃	++	++	+	+++	++++	+++	+++	−	++	++	−	−	−	+++
〃 8	〃	++	++	−	+	+++	+	+++	++	++	++	±	±	−	+++
〃 9	〃	++	++	−	+++	++++	+++	+++	−	++	++	−	−	−	++
〃 10	〃	++	++	−	++++	++++	++++	+++	±	++	++	−	−	−	±
〃 12	〃	++	++	±	++	++++	++++	+++	+	++	++	−	−	++	++
〃 21	〃	++	++	−	++	++++	++++	+++	++	++	++	±	±	−	+++
〃 22	〃	++	++	+	+++	++++	++++	+++	−	++	++	−	−	−	+++
〃 32	〃	++	++	−	−	++++	+	+++	++	++	++	−	−	−	+++
〃 33	〃	++	++	−	+++	++++	++++	+++	+	++	++	−	±	−	++
〃 36	〃	++	++	±	+++	+++	+++	+++	±	++	++	++	−	++	+++
〃 37	〃	++	++	+	++	++++	++	+++	+++	++	++	−	±	−	++
〃 39	〃	++	++	±	+	++++	++	+++	++	++	++	−	−	−	+++
〃 46	〃	++	++	−	+++	++++	++++	+++	++	++	++	−	+	−	+++
〃 51	〃	++	++	+	++++	+++	+++	+++	−	++	++	−	−	−	++++
〃 52	〃	++	++	±	+	++++	+++	+++	++	++	++	−	−	±	±
〃 66	〃	++	++	±	+	++++	+++	+++	+	++	++	−	−	±	±
〃 67	〃	++	++	++	++	++	++	+	−	++	++	−	−	++	+++
〃 68	〃	++	++	+	++	++++	+	++	+	++	++	−	−	++	+++
〃 69	〃	++	++	−	+	++++	++++	+++	++	++	++	−	−	−	+++
〃 71	〃	++	++	−	+	++++	++++	+++	++	++	++	−	−	−	+
〃 73	〃	++	++	−	+	+++	++	+++	++	++	++	−	−	−	+++
〃 75	〃	++	++	−	−	++++	++++	+++	++	++	++	−	−	−	+
〃 78	〃	++	++	±	+++	+++	++	++	±	++	++	−	±	−	+++
〃 83	〃	++	++	−	+++	++++	++	+++	±	++	++	−	−	−	+++
〃 87	〃	++	++	−	++	++++	++	+++	±	++	++	−	−	−	+++
〃 100	〃	++	++	−	+	++++	−	+++	++	++	++	−	±	−	+++
〃 107	〃	++	++	±	++	++++	++++	+++	−	++	++	−	−	−	++
〃 117	〃	++	++	−	++	++++	++++	+++	±	++	++	−	−	−	+++
〃 146	〃	++	++	−	+	+++	+	+++	++	++	++	−	±	−	+++
〃 161	〃	++	++	−	++	+++	++++	++	++	++	++	−	+	−	+++
〃 SZ*	〃	++	++	++	++++	++++	++	++	−	++	++	−	−	+	++
Penicillium corymbiferum	〃	++	++	−	−	++++	++++	+++	+++	++	++	−	+	±	++
Penicillium vinaceum	〃	++	++	−	+	++++	+++	+++	+++	++	++	+	+++	+	+
Penicillium notatum	〃	++	++	±	++	++++	+++	+++	++	++	++	−	+	−	++
Penicillium citrium	〃	++	++	−	±	++++	+	+++	++	++	++	−	++	−	++
Verticillium glancum	〃	++	++	±	−	++++	++++	+++	+++	++	++	−	−	−	−
Absidia glanca	〃	++	++	−	−	++++	++++	+++	+++	++	++	+	++	−	−
Absidia orchidis	〃	++	++	−	−	++++	++++	+++	+++	++	++	+++	+++	+	+
Mucor janseni	〃	++	++	−	−	++++	++++	+++	++	++	++	++++	−	−	+
Mucor circinelloides	〃	++	++	−	−	++++	++++	+++	+++	++	++	+++	+++	±	+
Oospora lactis	〃	++	++	−	−	++++	+++	+++	++	++	++	++++	++++	+++	++
Cunninghamella blakeslena	〃	++	++	−	−	++++	++++	+++	++	++	++	−	+	−	+
Cunninghamella echinulata	〃	++	++	−	−	++++	++++	+++	+++	++	++	−	−	−	−
Rhizopus peka	〃	++	++	−	±	++++	++++	+++	+++	++	++	−	+	−	−
Rhizopus javanicus	〃	++	++	−	+	++++	+++	+++	+++	++	++	+	+++	−	−
Cladosporium herbarum	〃	++	++	−	−	++++	++	+++	++	++	++	+	+	−	−
Pichia farinosa	〃	++	++	−	−	++++	++++	+++	++	++	++	−	++	−	−

* Isolates from "Tane-Koji" by the authors.

TABLE V. ACYLASE ACTIVITIES IN YEASTS

Organisms	Incubation	ε-Lysine Acylase Activity								α-Acylase Activity					
		Growth		Detected Amino Acids						Growth		Detected Amino Acids			
		(days)		Lysine (days)		ε-Acetyllysine (days)		ε-Benzoyllysine (days)		(days)		Methionine (days)		ε-Benzoyllysine (days)	
	°C	4	9	4	9	4	9	4	9	4	9	4	9	4	9
Saccharomyces cerevisiae	27	++	++	−	−	++++	++++	+++	+++	+	+	+	++	−	−
Saccharomyces peka	〃	++	++	−	−	++++	++++	+++	+++	+	+	−	±	−	−
Saccharomyces marxianus	〃	++	++	−	−	++++	++++	+++	+++	+	++	−	−	−	−
Saccharomyces partorianus	〃	++	++	−	−	++++	++++	+++	+++	+	++	+	+++	−	±
Saccharomyces fragilis	〃	++	++	−	−	++++	++++	+++	+++	±	+	+	+++	−	+
Saccharomyces logos	〃	++	++	−	−	++++	++++	+++	+++	+	+	±	+	−	−
Saccharomyces exignus	〃	++	++	−	−	++++	++++	+++	+++	±	+	−	−	−	±
Saccharomyces willianus	〃	++	++	−	−	++++	++++	+++	+++	++	++	±	+	−	−
Saccharomyces carlsbergensis	〃	+	++	−	−	++++	++++	+++	+++	+	+	−	+	−	−
Hansenula anomala	〃	++	++	−	−	++++	++++	+++	+++	++	++	+	+++	−	+
Pichia farinosa	〃	++	++	−	±	++++	++++	+++	+++	++	++	+	+	−	−
Torulopsis lactica	〃	+	++	−	−	++++	++++	+++	+++	+	+	+	++	−	−
Torula lactis	〃	++	++	−	−	++++	++++	+++	+++	+	++	−	±	−	−
Torula spp	〃	++	++	+	±	++++	++++	+++	+++	±	+	+	+++	±	+
Candida utilis	〃	++	++	±	−	++++	++++	+++	+++	+	+	−	+	−	−
Rhodotorula glutinis	〃	+	++	+	++	++++	++++	+++	++	+	+	+	++	−	±

On the screening for α-acylase activity in molds, although the spot of ε-benzoyllysine was detected in most of the tested molds, the expected methionine spot was scarcely detected. This result may be due to the consumption of formed methionine by living cells or due to an inadequate shift of pH for the activity during incubation.

Investigation of Yeasts—Although the occurrence of α-acylase in brewer's yeast had been already confirmed by the authers in a previous paper⁷⁾, in the experiments ε-lysine acylase and α-acylase activities of number of yeasts were examined.

As shown in Table V, a spot of lysine was detected only in the case of *Rhodotorula glutinis*, while other strains showed no distinct lysine spot.

On the other hand, the hydrolysis of acetylmethionine occurred in most of the yeasts tested, and ε-benzoyl-α-acetyllysine was, however, less susceptible.

DISCUSSION

In the present studies, various microorganisms have been screened to investigate ε-lysine acylase activity. For this purpose, the respective organisms were cultured on the medium containing ε-acyllsines, and ε-lysine acylase activity was estimated by the lysine spot and decrease in the spots of ε-acyllysines on paper chromatograms of the cultural broth.

As a result of the present study, the occurence of ε-lysine acylase activity in molds has been disclosed. Namely, in a number of the molds tested, especially in *Aspergillus oryzae*, the formation of a lysine spot and simultaneous decrease in the spots of ε-benzoyllysine were observed. Judging from the rate of decrease of the added ε-acyllysine, ε-benzoyllysine is supposed to be more susceptible to fungal ε-lysine acylase activity than ε-acetyllysine.

In other microorganisms such as bacteria, streptomyces and yeasts, the activity was scarcely detected under the conditions employed by the authors. In other words, ε-lysine acylase activity in organisms does not seem to be distributed so widely as α-acylase; this fact was confirmed by the same screening experiments simultaneously carried out as control experiments.

Under the conditions employed in the present screening test, it is uncertain that the media employed are most suitable to respective organisms for growth and enzyme formation. It is also possible that the amino acids formed by enzymatic hydrolysis are further metabolized by the organisms during incubation. Accordingly, under the different conditions, there is a possibility that organisms other than those detected by the present screening procedure may show higher ε-lysine acylase activity. Although, it is certain that the present method is simple and useful as a screening procedure to estimate rough tendency of the distribution of enzymatic activities.

Regarding the screening studies for α-acylase activity, acetylmethionine was hydrolyzed by most organisms except in the case of molds. As the wide distribution in molds of α-acylase activity hydrolyzing acetylmethionine has been already confirmed by the authors with enzymatic screening procedure[4)], the present method is considered to be an inadequate procedure for the screening of α-acylase activity of molds.

The present studies also reconfirmed the fact previously revealed by the authors, that is, although ε-benzoyl-α-acetyllysine is specifically readily hydrolyzed by the fungal acylase, the compound is less susceptible to acylase activities in other organisms. The only exception observed is the case of *Streptomyces rubrireticuli*, in which the spot of ε-benzoyllysine is evidently observed.

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Studies on Amino Acids. XIV.

On the ε-Lysine Acylase in Molds.

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For the purpose of investigating the formation of ε-lysine activity in molds, especially, *Aspergillus oryzae* was studied, from which it was found to be an inducible enzyme system whose activity is undetectable without induction.

The respective activities inducted with ε-benzoyl-L-, -D-, and -DL-lysines are capable of hydrolyzing both optical isomers of ε-benzoyllysine regardless of the optical nature of the inducers employed.

Several enzymatic properties of this fungal ε-lysine acylase are also presented.

In concern of ε-lysine acylase activity, that is, hydrolytic activity toward ε-N-acyllysine, Greenstein and his co-workers[1, 2] have first confirmed the occurrence of this activity in rat tissues. Kameda and his co-workers[3] have also studied on the enzyme with a strain of soil bacteria.

On the other hand, the present authors have reported on the distribution and enzymatic properties of acylase activities, that is, hydrolytic activities toward α-N-acyl amino acids, in animal sources as well as in molds[4,5], bacteria[6], yeast[7] and plants[8], also on its application to the resolutiou of racemic amino acids[5, 9~12].

In the foregoing paper[13], the authors have also reported on the distribution of ε-lysine acylase activity in microorganisms, and have revealed for the first time that the activity occurs in various kinds of molds.

In succession to the previous report, studies on the conditions of formation and enzymatic properties of ε-lysine acylase in molds, especially of *Aspergillus oryzae*, were performed and are presented in this paper. The most interesting fact disclosed by the present study is that this mold acylase differs from other previously known acylase activities observed in various sources, this ε-lysine acylase is an induced enzyme.

MATERIALS AND METHODS

Compounds Used—The preparation of ε-benzoyl-L-lysine and ε-acetyl-L-lysine has been described in a previous paper[13]. ε-Benzoyl-D-lysine was prepared by treating the copper complex of D-lysine with benzoylchloride. ε-Benzoylaminocaproic acid was prepared by the method of Galat[14], and ε-acetylaminocaproic acid by treatment of ε-aminocaproic acid with acetic anhydride in acetic acid solution.

Organisms—The organisms used were supplied

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Table I. Effect of Various Media Containing Malt Extract on the Formation of ε-Lysine Acylase Activity by *Aspergillus oryzae*

Media	ε-Benzoyl-L-lysine (mM)	Malt extract (°Bg.)	Glucose (%)	$(NH_4)_2SO_4$ (%)	Peptone (%)	$CaCO_3$ (%)	Method of culture	Enzyme Activity (μmole/hr.)*
No. 1	50	10/ 3	3.0	0.2	—	2.0	Stationary	1.4
No. 2	15	10/ 3	3.0	0.2	—	2.0	Stationary	2.1
No. 3	—	10/ 3	3.0	0.2	—	2.0	Stationary	0
No. 4	15	10/ 3	3.0	0.2	—	2.0	Shaken	2.8
No. 5	—	10/ 3	3.0	0.2	—	2.0	Shaken	0
No. 6	15	10/ 3	3.0	0.2	1.0	2.0	Stationary	0.9
No. 7	15	10/ 3	—	—	—	—	Shaken	2.4
No. 8	—	10/ 3	—	—	—	—	Shaken	0
No. 9	50	10/10	3.0	0.2	—	2.0	Stationary	0
No. 10	15	10/10	3.0	0.2	1.0	2.0	Stationary	0
No. 11	15	10/10	3.0	0.2	—	2.0	Stationary	0.6
No. 12	15	10/10	3.0	0.2	—	2.0	Shaken	0.6
No. 13	50	10/30	3.0	0.2	—	2.0	Stationary	0
No. 14	15	10/30	3.0	0.2	—	2.0	Stationary	0
No. 15	15	10/30	3.0	0.2	1.0	2.0	Stationary	0

The media used were adjusted to pH 7.5 with sodium hydroxide, and stationary culture was carried out for 6 days at 27° using 20ml of respective media in 100ml Erlenmeyer flasks. While shaken culture was carried out for 4 days at 27° using 50ml of respective media in 500ml shaking flasks. Enzyme assay was carried out under standard conditions with ε-benzoyl-L-lysine as the substrate.

* Enzyme activity in the extracts of the cells grown on a unit ml of the media.

through the courtesy of Dr. S. Fukui, Institute of Applied Microbiology, University of Tokyo.

Standard Enzyme Reaction—Unless otherwise noted enzyme reaction was carried out by incubating a mixture of 1 ml of enzyme solution, 1 ml of 0.01 M substrate solution and 1 ml of M/10 phosphate buffer (pH 8.2) at 37° for 1.5 hours. The liberated lysine was measured by Work's acidic colorimetric ninhydrin method[15, 16] with a minor modification. Namely, the ninhydrin reaction mixture was heated for 5 minutes and the coloured solution was diluted with 50% ethanol instead of acetic acid as employed in the original method. Protein nitrogen of the precipitate obtained from enzyme solution with 10% trichloroacetic acid was measured by the micro-Kjeldahl method. Enzyme activity was expressed in terms of micromoles of liberated lysine per mg of protein N or micromoles of liberated lysine per hour per g of wet cells, and total activity in micromoles of liberated lysine per hour.

Preparation of Cell-Free Extracts—Cells grown on respective media were harvested by centrifugation, washed twice with distilled water and weighed. The washed cells ground with quartz sand for 15~20 minutes in a mortar and extracted with M/50 phosphate buffer (pH 8.2) or with distilled water, weighing ten times of the wet cells. After standing in an ice box for 1~2 hours, the homogenate was centrifuged at 10,000×g for 25 minutes at 0°. The supernatant solution was used as cell-free extracts.

Enzyme Preparations—Enzyme preparations used in the studies on the enzymatic properties were obtained in the following manner. The medium consisting of malt extract (10°/3 Bg.) and 15 mM ε-benzoyl-L-lysine was adjusted to pH 7.0. This medium was distributed in amounts of 100 ml to 500 ml shaking flasks and was sterilized by autoclaving. After inoculation with two loopfuls of inoculum of *Aspergillus oryzae* No. 10, the cultures were incubated with reciprocal shaking (90 r.p.m.) at 30° for 4 days. The cells were harvested, ground and extracted with distilled water as previously described. The obtained extracts, enzyme preparation I, showed enzyme activity of 17.39 μmoles per hour per mg of protein-N by standard enzyme reaction employing ε-benzoyl-L-lysine as the substrate.

To the above extract (enzyme preparation I), ammonium sulfate was added to 0.4 saturation at pH 6.0. The resulting precipitate was collected with centrifugation, dissolved in distilled water, and dialyzed over-

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15) E. Work, *Biochem. J.*, **67**, 416 (1957).

16) F. P. Chinard, *J. Biol. Chem.*, **199**, 91 (1952).

night against cold distilled water in a cool place. The precipitate formed during dialysis was removed by centrifugation, and the supernatant solution was employed as enzyme preparation II. Enzyme activity of this preparation II was 40.45 μmoles per hour per mg of protein-N. This preparation was used in most experiments conducted on enzymatic properties.

RESULTS

Conditions of Culture for the Enzyme Formation—Since it was found from the previous screening test[13] that *Aspergillus oryzae* No. 10 shows a relatively higher ε-lysine acylase activity among the molds tested, conditions of culture for enzyme formation were examined as a preliminary test in order to investigate the properties of the enzyme.

Results of studies conducted on the effect of media containing varied amounts of malt extract, ε-benzoyl-L-lysine and some other supplements, on the formation of ε-lysine acylase activity by *Aspergillus oryzae* No. 10 are given in Table I. Since almost no activity was observed in the cultural broth in these experiments, it is assumed that the enzyme is endogenous under such conditions. Therefore the produced activities are compared by the enzyme activity in the extract of the cells produced by unit ml of the medium. The cell-free extracts were prepared according to the method previously mentioned.

As shown in the Table, enzyme activity was undetectable in the absence of ε-benzoyl-L-lysine in the medium and the concentration of malt extract was also influential for the formation of ε-lysine acylase activity. The results suggest that the presence of ε-benzoyl-L-lysine is indispensable for enzyme formation, in other words, ε-lysine acylase of *Aspergillus oryzae* No. 10 is supposed to be an adaptive enzyme.

Supplement of glucose, ammonium sulfate and calcium carbonate to malt extract, showed no distinct effect on the formation of enzyme activity as shown in experiments No. 4 and No. 7.

Besides the above non-synthetic media, the effects of various synthethic media and the presence of ε-benzoyl-L-lysine on the formation of ε-lysine acylase activity were also investigated.

TABLE II. EFFECT OF VARIOUS SYNTHETIC MEDIA ON THE FORMATION OF ε-LYSINE ACYLASE ACTIVITY BY *Aspergillus oryzae*

Media	Nitrogen source (%)	Carbon source (%)	Minerals (%)	ε-Benzoyl-L-lysine (mM)	Method of culture	Period of culture (days)	Enzyme activity (μ mole/hr.)*
Mayer	NH_4NO_3 1.0	Sucrose 5.0	KH_2PO_4 : 0.5 $MgSO_4\cdot 7H_2O$: 0.25 $CaHPO_4$: 0.25	0	Stationary	7	0
					Shaken	5	0
				15	Stationary	7	Trace
					Shaken	5	Trace
Henneberg	Peptone 1.0 KNO_3 0.2	Glucose 10.0	$NH_4H_2PO_4$: 0.2 $MgSO_4\cdot 7H_2O$: 0.05 $CaCl_2$: 0.01	0	Stationary	7	0
					Shaken	5	0
				15	Stationary	7	0.9
					Shaken	5	1.4
Czapek	$NaNO_3$ 2.0	Glucose 30.0	K_2HPO_4 : 1.0 $MgSO_4\cdot 7H_2O$: 0.5 KCl : 0.5 $FeSO_4\cdot 7H_2O$: 0.01	0	Stationary	6	0
					Shaken	4	0
				15	Stationary	6	0.8
					Shaken	4	4.4
Sabouraud	Peptone 1.0	Glucose 4.0	None	0	Stationary	6	0
					Shaken	4	0
				15	Stationary	6	6.8
					Shaken	4	3.0
Nageli	Ammonium tartrate : 1.0		K_2HPO_4 : 0.1 $MgSO_4\cdot 7H_2O$: 0.02 $CaCl_2$: 0.01	0	Stationary	6	0
					Shaken	4	0
				15	Stationary	6	Trace
					Shaken	4	Trace

Conditions of culture and enzyme assay were the same as described in Table I.
* Same as described in Table I.

From the results shown in Table II, it is apparent that the presence of ε-benzoyl-L-lysine is indispensable factor for enzyme formation, and higher activities are formed by both Czapek's media and Sabouraud's media than medium No. 4 containing malt extract.

Through the above experiments, somewhat better results were obtained by shaking culture than stationary culture expect in the case of Sabouraud's medium.

Besides the above experiments, previously induced *Aspergillus oryzae* No. 10, which had been successively cultured 4 times on medium No. 2 containing an inducer, was subsequently cultured on media No. 2 and No. 3, and then the enzymatic activity of the cells grown on the respective media were assayed. As the result, it was shown that neither occurence of activity in cells grown on medium No. 3 nor the increase in activity in the case of medium No. 2 by the previous induction is to be found. This result, like other most inductive systems, indicates that enzyme forming capacity in this microorganism can not be maintained after removal of the inducer from external environment.

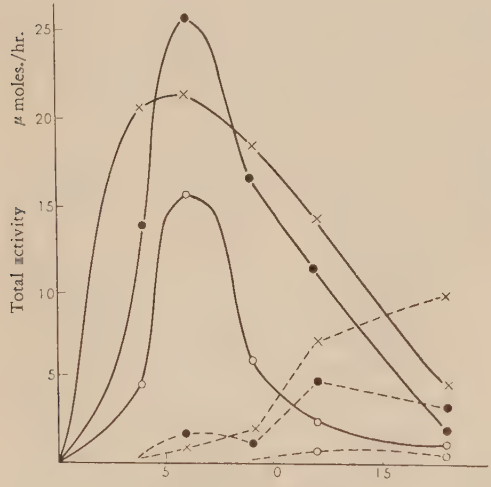

FIG. 1. Effect of Period of Culture and Concentration of the Inducer for Formation of ε-Lysine Acylase

The media used No. 3 medium in Table I supplemented with 10, 50, or 100 mM of ε-benzoyl-L-lysine, respectively. Stationary culture was carried out at 27° employing 20 ml of the medium in a 100 ml Erlenmeyer flask. Preparation of cell-free extracts and enzyme assay with ε-benzoyl-L-lysine as the substrate were carried out as previously described.

—— Activity in cells ---- Activity in media
○ 10 mM, ● 50 mM, × 100 mM

Effects of Period of Culture and Concentration of the Inducer for the Enzyme Formation

It is presumed from the previous expriments that ε-lysine acylase in *Aspergillus oryzae* No. 10 is an inducible enzyme. Thus, in order to clarify conditions dominating the induced enzyme synthesis, the effects of period of culture and concentration of the inducer were examined.

The basal medium employed for this experiment was medium No. 3 described in Table I. To the basal medium, ε-benzoyl-L-lysine was added at concentrations of 10, 50, or 100 mM. After inocubation of one loopful of *Aspergillus oryzae* No. 10, stationary culture was carried out at 27° and enzyme activities in cells and the broth were measured during incubation.

From the results, shown in Fig. 1, it is obvious that the enzyme activity in cells and total activity attain a maximum level after 6 days, and thereafter decrease with further incubation. In case of prolonged culture over 10 days, the appearance of enzyme activity in the cultural borth is recognized.

As for the effect of concentration of the inducer, as shown in Fig. 1, apparently higher activity was found in the case of a 50 mM concentration of ε-benzoyl-L-lysine than that of a 10 mM concentration, although, no significant difference was observed between concentrations of50 mM and 100 mM. As shown in experiments No. 1, and No. 2 of Table I, a slightly higher activity than that of 50 mM was formed in the case of a 15 mM concentration. Therefore the addition of an inducer at a concentration of 15 mM was employed for the most experiments of induction.

Effect of Inducer for the Specificity of the Enzyme

Acylase activity, namely hydrolytic activity toward α-N-acylamino acids, which occurs in animals and micro-organisms, has optical specificity and hydrolyzes only α-N-acyl-L-amino acids with the exception of a certain soil bacterium. Also, ε-lysine acylases in animal tissues and in soil bacteria as reported by Greenstein[1, 2] and Kameda[3], respectively, have optical specificity on the hydrolysis of ε-N-acyl-DL-lysines.

In order to study the optical specificity of fungal ε-lysine acylase, hydrolytic activities ε-benzoyl-L- and -D-lysines of the cell-free extracts prepared from the molds grown on the medium containing ε-benzoyl-L-lysine, were examined. As shown in Table III, an interesting result was obtained, that is, unlike previously known ε-lysine acylases, the activity formed in molds shows no optical specificity and hydrolyzes both optical isomers.

TABLE III. OPTICAL SPECIFICITY OF ε-LYSINE ACYLASE IN CELL-FREE EXTRACTS OF MOLDS

Molds	Period of culture days	Total enzyme activity (μ mole/hr.) ε-Benzoyl-L-lysine	ε-Benzoyl-D-lysine
Aspergillus flavus	7	50.8	64.3
Aspergillus oryzae No. 9	6	8.3	10.2
Aspergillus oryzae No. 10	6	79.0	99.7
Aspergillus oryzae No. 51	6	20.6	28.2
Aspergillus oryzae SZ	7	38.2	45.2

Stationary culture was carried out for 6 or 7 days at 27° using 40 ml of the medium (No. 1 in Table I) in 200 ml Erlenmeyer flasks. Preparation of cell-free extracts was previously mentioned, and enzyme assay was carried out under standard conditions with ε-benzoyl-L-lysine and ε-benzoyl-D-lysine as the substrates.

Besides the above mentioned substrates, hydrolytic activity toward ε-acetyl-L-lysine, ε-benzoylamino caproic acid and ε-acetylamino caproic acid were also studied with the extracts of the cells of *Aspergillus oryzae* No. 10 inducted with ε-benzoyl-L-lysine. As a result, ε-benzoylamino caproic acid was found to be hydrolyzed at pH 8.0 with a half-rate of the hydrolysis of ε-benzoyl-L-lysine, while the other substrates tested were insuceptible. When the extracts of the cells of *Aspergillus oryzae* No. 10 cultured without induction of ε-benzoyl-L-lysine were employed, hydrolysis of ε-benzoyl-amino caproic acid was not observed.

Further investigations employing *Aspergillus oryzae* No. 10 were carried out to decide whether optical specificity is affected with the optical properties of inducers or not. The resuls, shown in Table IV, indicate that ε-benzoyl-L-, -D-, and -DL-lysines are capable of inducing ε-lysine acylase synthesis, and respective activities formed act on both optical isomers, ε-benzoyl-L- and -D-lysines.

TABLE IV. EFFECT OF INDUCERS ON THE OPTICAL SPECIFICITY OF THE ENZYME

Inducers	Total enzyme activity (μ mole/hr.) ε-Benzoyl-L-lysine	ε-Benzoyl-D-lysine
None	0	0
ε-Benzoyl-L-lysine	419	617
ε-Benzoyl-D-lysine	199	319
ε-Benzoyl-DL-lysine	678	818

Shaken culture was carried out for 4 days at 27° with 500 ml shaking flasks containing 100 ml of the medium, which composed of malt extract (10°/3 Bg.) and 15 mM of ε-benzoyl-L-, -D-, or -DL-lysines as the inducer and adjusted to pH 7.5. Preparation of cell-free extracts was previously mentioned, and enzyme assay was carried out under standard conditions with ε-benzoyl-L-lysine or ε-benzoyl-D-lysine as the substrate.

Properties of the Enzyme

From the above experiments it was confirmed that fungal ε-lysine acylase induced by ε-benzoyl-L-lysine hydrolyzes ε-benzoyllysine without distinction of the optical difference of the substrate. To ascertain whether the hydrolytic activities toward both optical isomers of ε-benzoyllysine is due to the action of a single enzyme or due to the presence of separate enzymes which may be called "ε-L-lysine acylase" and "ε-D-lysine acylase", enzymatic properties of the induced ε-lysine acylase system were investigated with the preparations obtained from *Aspergillus oryzae* No. 10.

Fractionation of Enzyme Solution with Ammonium Sulphate and Acetone—The cell-free extracts prepared according to the method previously mentioned were fractionated with ammonium sulfate and acetone. Activities of the respective fractions were measured and the results are summarized in Table V. The ratios of the rates of hydrolysis of the two isomers are not changed by these fractionation procedures.

Effect of Heat Treatment—In Table VI, the influence of heat treatment on the activities

TABLE V. FRACTIONATION WITH AMMONIUM SULPHATE AND ACETONE

Fraction	Enzyme activity*		L/D**
	ε-Benzoyl-L-lysine	ε-Benzoyl-D-lysine	
E_1 : Cell-free extracts	23.9 (17.39)	26.9 (19.51)	0.89
E_2 : 0～0.4 satu. with $(NH_4)_2SO_4$ of E_1	11.9 (40.45)	15.9 (53.88)	0.75
E_3 : 45～70% with acetone of E_2	11.8 (189.6)	14.1 (225.3)	0.84
E_4 : 0～50% with acetone of E_1	2.8	3.5	0.80
E_5 : 50～70% with acetone of E_1	5.1	6.1	0.84

* Micromoles of lysine liberated per hour per g of wet cells. The figures in brackets show μmoles per hour per mg of protein N.

** L/D: Ratio of rates of hydrolysis of ε-benzoyl-L-lysine and ε-benzoyl-D-lysine.

Enzyme assay was carried out under standard conditions with ε-benzoyl-L-lysine and ε-benzoyl-D-lysine as the substrates.

TABLE VI. EFFECT OF HEAT TREATMENT

Temperature and period of heat treatment	Relative Activity		L/D
	ε-Benzoyl-L-lysine	ε-Benzoyl-D-lysine	
No treatment	100*	100**	0.88
45°, 5 mins.	31	31	0.89
45°, 10 mins.	13	15	0.76
50°, 5 mins.	0	0	—

* 17.4 μmoles per hour per mg of protein N.

** 19.4 μmoles per hour per mg of protein N.

Eyzyme preparation I, previously mentioned, was used for the experiments. Enzyme assay was carried out under standard conditions with ε-benzoyl-L-lysine and ε-benzoyl-D-lysine as the substrates.

The activity of the respective substrate without heat treatment was taken as 100.

TABLE VII. EFFECT OF pH AND BUFFERS ON THE ENZYME ACTIVITY

Buffers	pH	Enzyme Activity*		L/D
		ε-Benzoyl-L-lysine	ε-Benzoyl-D-lysine	
Phosphate	5.0	0	0	—
	6.0	7.6	10.2	0.75
	7.0	11.2	12.9	0.87
	8.2	40.5	54.3	0.75
	8.6	34.8	43.0	0.81
Ammonia and ammonium chloride	8.0	15.4	17.4	0.89
	8.4	34.4	45.2	0.76
	8.9	24.4	32.0	0.76
	9.6	20.2	27.2	0.74
	10.4	19.7	24.3	0.81
Veronal	6.9	13.4	15.7	0.80
	8.3	28.8	40.4	0.71
	9.1	27.1	37.0	0.73
Tris (hydroxymethyl)-aminomethane	6.9	10.5	—	—
	7.6	17.8	—	—
	8.2	34.4	45.2	0.76
	8.5	33.1	40.9	0.81
	9.1	32.4	39.4	0.82

* Micromoles of lysine liberated per hour per mg of protein N.

Enzyme preparation II, previously mentioned, was used, and assay was carried out under standard conditions except for the buffer employed.

toward ε-benzoyl-L- and -D-lysines is shown. As it is revealed from the Table VI, the decrease in activity with time toward the two substrates was essentially parallel, and treatment at 50° for 5 minutes completely abolished the activities.

Effect of pH and Buffers—Effect of pH and buffers on enzyme activity was studied, and the results are presented in Table VII. Change of optimal pH due to the difference of buffers was not observed, and optimal pH for hydrolysis of both isomers was found to be around 8.2～8.5 in phosphate, ammonium, veronal and tris-(hydroxymethyl)-aminomethane buffers employed, and phosphate buffer showed the highest activity.

Throughout the experiments, no significant change in the ratios of the activities toward ε-benzoyl-L- and -D-lysines was observed in respective pH and buffers.

Effect of Metals and Inhibitors—In order to study the influence of metals and inhibitors, various metal salts, sulfhydryl reagents, metal binders and other inhibitors were added to the enzyme assay, and their activities were compared by employing both optical isomers of ε-benzoyl-lysine as the substrates.

As summarized in Table VIII, zinc acetate showed complete inhibition of the enzyme action, and salts of copper and nickel also acid inhibitory. Although a slight stimulation was observed by the addition of strontium salt, other metals

TABLE VIII. EFFECT OF METALS AND INHIBITORS ON THE ENZYME ACTIVITY

Additions	Relative activity		
	ε-Benzoyl-L-lysine	ε-Benzoyl-D-lysine	L/D
None	100*	100**	0.76
$CaCl_2$	102	103	0.74
$CoCl_2$	84	72	0.88
$CuCl_2$	56	49	0.83
$FeSO_4 \cdot (HN_4)_2SO_4$	82	82	0.76
$MgCl_2$	99	100	0.76
$MnCl_2$	110	103	0.81
$NiCl_2$	43	38	0.88
$SrCl_2$	134	129	0.79
$(CH_3COO)_2Zn$	0	0	—
NaCN	114	111	0.78
NaF	59	64	0.70
Ethylenediaminetetraacetate	113	121	0.71
Iodoacetate	102	102	0.76
p-Chloromercuribenzoate	9	0	—

* 34.4 μmoles lysine liberated per hour per mg of protein N.

** 45.2 μmoles lysine liberated per hour per mg of protein N.

The incubation mixture contained 1 ml of 0.01M substrate solution, 0.5 ml of enzyme preparation II solution, 1 ml of M/10 tris buffer (pH 8.2), and 0.5 ml of 6×10^{-3}M solution of the addition (final concentration of the addition was 1×10^{-3}M except for *p*-chloromercuribenzoate which was 0.5×10^{-3}M). Liberated lysine was determined by the method previously mentioned. The activity of the respective substrate without addition was taken as 100. A little amount of precipitate was formed when salts of copper, nickel, strontium and zinc were added to the enzyme reaction mixture.

showed no activation of activity under the conditions employed.

Although sodium fluoride was inhibitory, the addition of sodium cyanide and ethylenediamineacetate showed a slightly enhancing effect on the enzymatic hydrolysis. In the case of sulfhydryl reagents, complete inhibition was observed with *p*-chloromercuribenzoate, however, iodoacetate showed no inhibitory effect.

On the rates of hydrolysis of the two substrates, similar effects were observed throughout the experiments by the addition of metals and inhibitors.

DISCUSSION

Recently the occurrence of ε-lysine acylase activity, that is, hydrolytic activity toward ε-N-acyllysine, was confirmed in animal tissues and in a soil bacterium. On the other hand, in the previous paper[13] the authors have first disclosed the presence of the activity in molds.

In succession to the previous report, conditions of enzyme formation and enzymatic properties of ε-lysine acylase activity in *Aspergillus oryzae* were studied. As a result, fungal ε-lysine acylase was found to be an adaptive enzyme, that is, the activity is inducibly formed under the presence of ε-benzoyllysine. This finding characterizes fungal ε-lysine acylase from other previously known acylases.

Although previously reported ε-lysine acylases in rat kidney and in a soil bacterium have optical specificity and solely act upon ε-acyl-L-lysines, respective activities formed in *Aspergillus oryzae* by the induction with ε-benzoyl-L-, -D-, and -DL-lysines could hydrolyze not only L-isomer but also D-isomer of ε-benzoyllysine at a rather faster rate than that of the former. These data at least suggest two possibilities. The first is that the induced enzyme has no optical specificity on the hydrolysis of both optical antipodes. The second possibility is that induction results formation of two separate enzymes, one of which acting upon benzoyl-L-lysine, whereas the other acting on benzoyl-D-lysine.

Through the present studies on the properties of this inducible fungal ε-lysine acylase including differential fractionation, heat stability, the relation of activity and pH in various buffer solutions, activation and inhibition experiments, and comparison of the rates of hydrolysis toward L- and D-isomers of ε-benzoyllysine, no appreciable change of ratio was observed. Although the results of these indirect approaches do not completely eliminate the possibility of the presence of two hardly separable enzymes, the results obtained are in favor of the possibility that hydrolysis of the both optical isomers of ε-benzoyllysine is due to an identical enzyme system.

Besides both optical antipodes of ε-benzoyllysine, fungal enzyme system inducted with ε-benzoyl-L-lysine is capable of hydrolyzing ε-benzoylamino caproic acid. In spite of this, ε-acetyl-L-lysine, which is susceptible to renal ε-lysine acylase, and ε-acetylamino caproic acid were not hydrolyzed by this fungal system under the conditions employed by the authors.

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[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 1, p. 44～51, 1960]

Modified Starch. I.

Physicochemical Properties: On Infrared Absorption Spectra of Native Starch and Modified Starch using Film

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The infrared absorption spectra of native (potato) and new modified starches were observed in about 4500 cm^{-1}～670 cm^{-1} region using film technique as a new attempt. Deuteration was attempted to investigate the physicochemical properties and to assign the absorption bands partially. And the absorption band attributed to the H_2O molecule was explained. The absorption band, which appeared in the modified starch only and which was thought as one great difference between two starches seems to be related to CO.

INTRODUCTION

Although the infrared spectra of high polymers have been reported, its interpretation has been very difficult. As a result of the use of extended spectroscopic data and group theoretical method of analysis, a more detailed and complete interpretation of the infrared spectra of several high polymers, polyvinyl alcohol, polyethylene, cellulose, or peptide etc., has been achieved. Especially, the investigation on infrared spectrum of cellulose relating mostly to starch has been published by many researchers. But, many reports on starch could not be found. Particularly, the infrared spectroscopic investigation using film technique has not been published. Using film technique as a new attempt, the author investigated the physicochemical properties of native and new modified starches. It is the purpose of this paper to present the results of further spectroscopic studies on both the starches, and to make some tentative assignments of the bands in infrared spectra on the basis of data of polyvinyl alcohol, polyethylene, and cellulose. The results of the comparison of the physicochemical properties of both the starches were described. In the other paper, the comparison of the physicochemical properties of both the starches with infrared spectroscopy using KBr-disk will be published.

The films of native and modified starches were deuterated in order to investigate the effect of deuteration that contributed to ascertain those assignments of the absorption bands in the in-

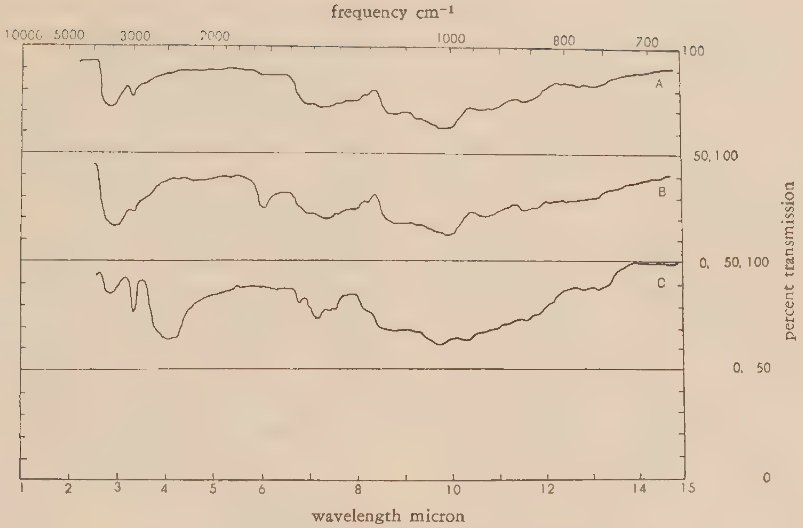

FIG. 1. Infrared Spectra of Native Starch

(A) undeuterated, dried. (B) hydrated, wet. (C) deuterated, wet.

frared spectra*. And this experiment seems to be suitable for qualitative analysis or somewhat quantitative analysis. On the other hand, the results obtained from infrared spectroscopy using KBr-disk were ascertained with the deuteration.

EXPERIMENTAL

Native starch The starch used was potato starch, potato was obtained from the farm of the University of Tokyo. It was purified carefully with the methed of Schoch[1].

Modified starch The modified starch was prepared from starch and formamide by heating. 30 g of the above purified potato starch was suspended in 300 g of formamide. Ascertaining the homogeneity of suspension, this reaction was continued for 6¼ hours at 140°C. The filtered cooled colloidal solution was poured into 900ml of methanol. Next day, the white soft reaction product was filtered, and was washed with a suitable quantity of methanol and absolute ethanol, and was dried under reduced pressure. This modified starch was hygroscopic. The yield was 27.5 g.

Film a) native starch ; weighed amount of the above-described native starch was suspended in water, and a thin film was formed from the suspension on a AgCl plate. The weight per aera was 0.027mg/mm^2. **b)** modified starch ; weighed amount of the above-described modified starch was dissolved into water, and a thin film was formed from the colloidal solution on a AgCl plate. In order to investigate the properties of modified starch, the ratio of weight per area was changed to 0.019 mg/mm^2. (The light scattering power of native starch was larger than that of modified starch.)

Deuteration The deuteration was accomplished by bringing these films into D_2O vapor. The samples, which were formed to film state on a AgCl plate, were mounted in a humidity controlling infrared absorption cell. At first, the humidity in the cell was kept at about 75 % by $NaClO_3$ saturated H_2O solution which was placed on the bottom of the cell. At the same time, the solid of $NaClO_3$ must be kepted completely on the state of supersaturation. Its humidity was more suitable to this object than the about 44% or 64%. In the relation of absorption intensity to time, about 20 hours were sufficient for the saturation. But, by way of precaution, the determination was undertaken after 22∼24 hours in all cases. These spectra were shown in Fig. 1–(B) for native starch and Fig. 2–(B) for modified starch respectively. Next, the humidity was brought nearly to zero by replacing the above solution by concentrated H_2SO_4 ($d=1.83$) and the film was dried for 22∼24 hours by checking the absorption intensity.

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* The experiments using film of native, modified, pre-hydrolyzed, and pre-hydrolyzed modified starches were undertaken one year ago. Soon after, the above four experiments using film were undertaken, the apparatus was partially changed.

1) T. J. Schoch, *J. Am. Chem. Soc.*, **64**, 2957 (1942).

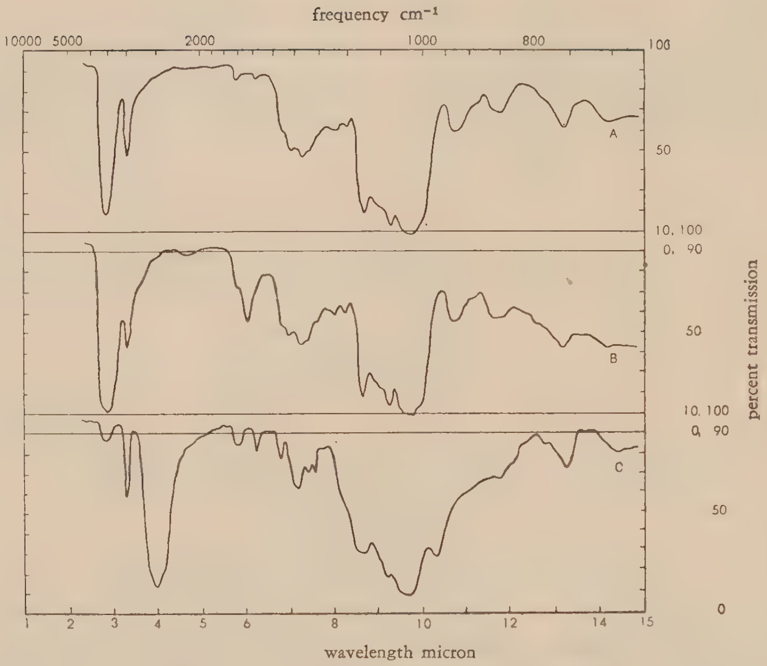

FIG. 2. Infrared Spectra of Modified Starch

(A) undeuterated, dried. (B) hydrated, wet. (C) deutrated, wet.

These spectra were shown in Fig. 1–(A) for native starch and Fig. 2–(A) for modified starch respectively. Then, the above H_2SO_4 was replaced by $NaClO_3$ saturated D_2O solution without contacting the atmospheric H_2O vapor, really the exchange was carried out in the dry box using molecular sieve. The film was deuterated by this solution through vapor phase exchange. Of course, the D_2O solution must be the solution with the solid phase on the bottom. After 22∼24 hours, the absorption intensity was determined. In the half way, the absorption intensity was determined to check the change against time. These spectra were shown in Fig. 1–(C) for native starch and Fig. 2–(C) for modified starch respectively. On the other hand, in the exchange H_2O for D_2O, its exchange rate was rather large in both the starches. In the exchange D_2O for H_2O, the reversibility was recognized in both the starches, but in the exchange rate, modified starch seems to be larger than native starch by somewhat quantitative analysis.

Apparatus Koken DS 301 type Infrared-spectro-recording Photometer with NaCl optics was used. The infrared absorption spectra of the both starches using film technique were determined in the range of about 4500 cm^{-1} to about 670 cm^{-1}.

TABLE I. BANDS CHANGES RESULTING FROM MODIFICATION

(dried state, ( ) is native starch)

change				unchange	
3394		1089		2921	(2927)
	(3369)	1083		1461	(1468)
1721			(1076)	1418	(1418)
1696		1023		1403	(1396)
1591			(1014)	1372	(1370)
1439			(984)	1342	(1349)
1383		937		1311	(1311)
	(1360)		(865)	1253	(1249)
	(1330)	855		1205	(1200)
	(1292)	758		931	(934)
1239		706		921	(917)
	(1214)			851	(847)
1151				793	(789)
	(1131)			773	(771)

The comparisons of dried modified and native starches (Fig. 2–(A) and Fig. 1–(A)), hydrated (undeuterated, wet) modified and native starches (Fig. 2–(C) and Fig. 1–(C)), deuterated and hydrated native starches (Fig. 1–(C) and Fig. 1–(B)), and, deuterated and hydrated modified starches (Fig. 2-(C) and Fig. 2-(B)) were considered. Then, the data resulting from the above comparisons were summarized in Table I to Table V respectively. These frequencies were calibrated with polystylene.

In Table I, the band near 3370 cm^{-1} changed slightly, but the band near 2945 cm^{-1} did not change. The band relating to adsorbed H_2O disappeared. The new bands in the region 1720 ∼ 1700 cm^{-1} and near 1590 cm^{-1} appeared only in the spectrum of modified starch like the spectrum using KBr-disk. The bands in the region 1470 ∼ 1050 cm^{-1} did not change greatly. The strongest band of modified starch appeared at 1023 cm^{-1}, and that of native starch appeared at 1014 cm^{-1}. In the low frequency region, several changes were observed.

TABLE II. BANDS CHANGES RESULTING FROM MODIFICATION

(hydrated state, ( ) is native starch)

change				unchange	
3350			(1318)	2919	(2906)
	(3328)	1306		1450	(1454)
1701		1149		1426	(1430)
1639			(1135)	1408	(1409)
	(1622)		(1068)	1353	(1356)
1589		1050		1333	(1335)
	(1462)	1021		1242	(1247)
1458			(1003)	1207	(1206)
1418			(981)	919	(915)
	(1392)	939		864	(861)
1382			(929)	800	(801)
1365		853		760	(762)
	(1346)		(845)		
			(815)		
		786			
		706			

In Table II, the band near 3370cm^{-1} changed slightly. The band near 2910 cm^{-1} was unchanged. The new bands of modified starch appeared near 1700 cm^{-1} and near 1590 cm^{-1}. The band relating to adsorbed H_2O appeared near 1640 cm^{-1}. The bands in the region 1470 ∼ 1050 cm^{-1} did not change greatly. The strongest band of modified starch appeared at 1021 cm^{-1}, and that of native starch appeared at 1003 cm^{-1}. From the data of Table I and Table II, the strongest band appeared at slightly different frequency. Perhaps, those bands may be shifted very little by hydration. In the low frequency region, the bands near 930, 843 and 760cm^{-1} changed slightly as compared with the data in Table I.

TABLE III. BANDS CHANGES RESULTING FROM MODIFICATION

(deuterated state, ( ) is native starch)

change				unchange	
3406			(1014)	2921	(2915)
	(3352)	971		2461	(2468)
1701			(962)	1462	(1462)
1681			(940)	1419	(1419)
1589		929		1382	(1389)
1423			(911)	1336	(1332)
	(1401)		(887)	1305	(1314)
	(1238)		(861)	1286	(1284)
1159		851		1205	(1208)
	(1125)		(843)	757	(761)
1076		785			
	(1068)		(779)		
1029		697			

In Table III, the band in the region 3400 ∼ 3350cm^{-1} changed slightly. The band near 2920 cm^{-1} did not change. The new bands appeared in the region 2480 ∼ 2450 cm^{-1} (in modified starch), and in the region 2470 ∼ 2390 cm^{-1} (in native starch). The bands in the region 1705 ∼ 1680 cm^{-1} and near 1590 cm^{-1} appeared only in modified starch. The bands in the region 1420 ∼ 1050cm^{-1} did not change greatly. The strongest band of modified starch appeared at 1029 cm^{-1}, and that of native starch appeared at 1014 cm^{-1}. The new bands appeared in the region 970 ∼ 960 cm^{-1}. In the region 850 ∼ 670cm^{-1}, several changes were observed, the band near 785 cm^{-1} appeared distinctly in modified starch.

TABLE IV. BANDS CHANGES RESULTING FROM DEUTERATION IN NATIVE STARCH

(( ) is hydrated specimen)

change				unchange	
3352			(1135)	2915	(2906)
	(3328)	1125		1462	(1462)
2468		1014		1389	(1392)
	(1622)		(1003)	1332	(1335)
	(1454)		(981)	1314	(1318)
	(1430)	962		1208	(1206)
1419		940		1064	(1068)
	(1409)		(929)	911	(915)
1401		887		861	(861)
	(1356)		(815)	843	(845)
	(1346)		(801)	761	(762)
1284		779			
	(1247)				
1238					

In Table IV, the band in the region 3350~3330 cm^{-1} weakened considerably. The band near 2910 cm^{-1} weakened without shift. In the region 2450~2300 cm^{-1}, the new band appeared distinctly on deuteration. The band relating to adsorbed H_2O near 1620 cm^{-1} disappeared. In the region 1460~1235 cm^{-1}, main bands (7 bands) weakened generally. The stronger band appeared in the region 1210~1150 cm^{-1}. The bands in the region 1150~1050 cm^{-1} did not change greatly. The strongest band shifted from 1003 cm^{-1} to 1014 cm^{-1}. The new stronger band appeared near 960 cm^{-1} on deuteration. The stronger bands in the region 940~845 cm^{-1}. The bands in the region 830~670 cm^{-1} weakened.

TABLE V. BANDS CHANGES RESULTING FROM DEUTERATION IN MODIFIED STARCH
(( ) is hydrated specimen)

change				unchange	
3406		1029		2921	(2919)
	(3350)		(1021)	1701	(1701)
2461		971		1589	(1589)
	(1639)		(939)	1462	(1458)
	(1450)	929		1423	(1426)
	(1408)		(919)	1419	(1418)
	(1365)		(864)	1382	(1382)
	(1354)		(800)	1336	(1333)
1286			(706)	1305	(1306)
	(1242)	697		1205	(1207)
1159				851	(853)
	(1149)			785	(786)
1076				757	(760)
	(1050)				

In Table V, the band in the region 3400~3350 cm^{-1} weakened considerably. The band near 2920 cm^{-1} weakened without shift. In the region 2480~2445cm^{-1}, the new band appeared distinctly on deuteration. The band in the region 1705~1680 cm^{-1} weakened without large shift. The band relating to adsorbed H_2O near 1640 cm^{-1} disappeared. The band near 1590 cm^{-1} weakened without shift. In the region 1470~1235 cm^{-1}, main bands (7 bands) weakened generally. The stronger band appeared in the region 1210~1170 cm^{-1}. The bands in the region 1160~1040 cm^{-1} weakened. The strongest band shifted from 1021 cm^{-1} to 1029 cm^{-1}. The new stronger band appeared near 970 cm^{-1} on deuteration. The bands in the region 950~670 cm^{-1} weakened. The band near 800 cm^{-1} weakened especially and the band near 785 cm^{-1} weakened distinctly.

Before attempting the assignments of the bands in the infrared spectrum, it is appropriate that the author assumed what frequencies may be expected to appear. It can be done most effectively by means of a group theoretical approach in the future. In the present case, the assignments of the bands in the infrared spectra of native and modified starches were attempted by comparison with the infrared spectra of polyvinyl alcohol, polyethylene and cellulose etc. In the assignments of various atomic groups, the vibrations of the CH and OH groups will be considered together, since some of vibrations of the CH and OH groups appears to interact each other. But in this case, (1) deuteration, (2) CH and CH_2 groups, (3) OH group and (4) CO and CC groups were considered.

RESULTS

(1) **Deuteration** Before going into a comparison of hydrated (undeuterated, wet) and deuterated films, the absorption band relating to H_2O molecule adsorbed by the film was mentioned at first. In dried state, the intensity of the band near 3350 cm^{-1} was reduced to about 56% (transmission/weight) of its original intensity (ca. 75% humidity) in native starch, and that was reduced to about 57% (transmission/weight) of its original intensity (ca. 75% humidity) in modified starch. The band in the region 1640~1625 cm^{-1} disappeared in both the specimens. Therefore, those absorptions were attributed to the H_2O molecule in both the hydrated starches.

In these deuterated films, the intensity near 3350 cm^{-1} was reduced markedly to about 24% (transmission/weight, in native starch), and about 8.4% (transmission/weight, in modified starch). This difference may be due to one difference of the physicochemical properties between the both starches. And the difference may cause to modify starch. The band near 2910 cm^{-1} (in native and modified starches) due to CH group remained unchanged like cellulose (the band may include the splitable band relating to CH_2 group). The strong OD band appeared near 2465 cm^{-1} (in native starch), near 2460 cm^{-1} (in modified starch). In the low frequency region, some decrease in intensity was observed throughout the region 1550~1250 cm^{-1} (in native and modified starches) and some strong bands were observed in the region 1160~950 cm^{-1} (in native and modified starches). The bands in the region 1550~1250 cm^{-1} may be related to CO streching vibration. The bands at 1454, 1356 cm^{-1} (in native starch) and at 1450, 1354 cm^{-1} (in modified starch) weakened especially, at the same time, the strong bands appeared at 1389 cm^{-1} (in native starch) and at 1382 cm^{-1} (in modified starch). These changes seem to be similar to the cases of aliphatic alcohols[2] and polyvinyl alcohol[3,4] deuterated. In polyvinyl alcohol, the two peaks at 1446 and 1326 cm^{-1} disappeared on deuteration. But the new strong band appeared at 1384 cm^{-1}. This band was attributed to pure CH deformation and the two peaks were ascribed to the coupling of the OH and CH deformation motions. Then, the bands at 1454, 1356 cm^{-1} (in native starch), and 1450, 1354 cm^{-1} (in modified starch) may be ascribed to the coupling of the OH and CH deformation motions, the band at 1389 cm^{-1} (in native starch), and at 1382 cm^{-1} (in modified starch) may be related to the CH bending vibration (+CH$_2$ wagging). In the region 1550~1250 cm^{-1}, the bands at 1462, 1335 and 1318 cm^{-1} (in native starch), and 1458, 1333 and 1306 cm^{-1} (in modified starch) seem to remain unchanged on deuteration, the band at 1462, 1458 cm^{-1} may be related to the CH$_2$ bending vibration, the bands at 1335, 1318, and 1333, 1306 cm^{-1} may be related to the CH bending vibration, (+CH$_2$ wagging). In the case of cellulose[5], the bands at 1426, 1365, 1310 and 1275 cm^{-1} may be ascribed to the CH deformation vibrations. The bands at 1247, 1206 cm^{-1} (in native starch) and at 1242, 1207 cm^{-1} (in modified starch) were observed in the undeuterated films. The strong band appeared in the region 1250~1120 cm^{-1} on deuteration. These bands may be related to the CH$_2$ wagging, CH bending, OH deformation vibrations (+CH$_2$ twisting). In the case of cellulose, the bands at 1275, 1249, 1232 and 1204 cm^{-1} were assigned to the CH$_2$ wagging, CH and OH deformation vibrations. Lower the frequency, the more difficult the assignment becomes. The bands at 1135, 1102 and 1068 cm^{-1} (in native starch), and at 1149, 1103 and 1050 cm^{-1} (in modified starch) weakened with little shift partially. The strongest band at 1003 cm^{-1} (in native starch), and at 1021 cm^{-1} (in modified starch) shifted to 1014 cm^{-1} (in native starch), to 1029 cm^{-1} (in modified starch) on deuteration. The band at 1135 cm^{-1} (in native starch), at 1149 cm^{-1} (in modified starch) may be related to the COC streching vibration. The bands at 1102, 1068, 1003 cm^{-1} (in native starch) and at 1103, 1050, 1021 cm^{-1} (in modified starch) may be related to the CO, CC streching vibrations. In the case of cellulose, the bands at 1161, 1117, 1106, 1078, 1069, 1030 and 1006 cm^{-1} were assigned to the CO, CC streching and CH$_2$ rocking vibrations. (In polyvinyl alcohol, the band at 1144 cm^{-1} may be the COC streching vibration.) The new band appeared at 962 cm^{-1} (in native starch), at 971 cm^{-1} (in modified starch) on deuteration, the band may be related to the OD deformation vibration. In the case of the deuterated polyvinyl alcohol, the band at 985 cm^{-1} was ascribed to the OD deformation vibration. In both the starches, the band near 670 cm^{-1} may be related to the OH out of plane deformation vibration. In the case of cellulose, the band near 650 cm^{-1} was assigned to the OH out of plane deformation mode.

(2) **CH and CH$_2$ groups** The spectral feature in the region 3000~2800 cm^{-1} is probably determined largely by the contribution from the CH streching vibration and also partially from the CH$_2$ streching vibration of the CH$_2$OH group in each glucose residue. The band near 2905 cm^{-1} (in native starch), and near 2920 cm^{-1} (in modified starch) may be related to the CH and CH$_2$ streching vibrations. The bands in

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the region 1500～1400 cm^{-1}, ca. 1460 cm^{-1} (polyethylene)$^{6)}$, 1430 cm^{-1} (polyvinyl alcohol)$^{7)}$, 1426 cm^{-1} (cellulose) were assigned to the CH_2 bending vibration.

The band at 1462 cm^{-1} (in native starch), at 1458 cm^{-1} (in modified starch) remained unchanged on deuteration. So, the band may be related to the CH_2 bending vibration. The difference between the various polymers may reflect the slightly different force field for this vibration. The intensity of the band relating to the CH_2 wagging mode or CH_2 twisting mode seems to be some weak. Moreover, the assignment of the CH_2 rocking mode seems to be more difficult than that of the other CH_2 modes. The band relating to the CH_2 rocking vibration seems to be expected in the range of 1000～700 cm^{-1}$^{8)}$. In the case of polyvinyl alcohol, there were two such bands at 917 and 851 cm^{-1}, the band at 917 cm^{-1} was assigned to the CH_2 rocking mode on the basis of relative intensity considerations. The bands at 929, 915, 845 cm^{-1} (in native starch), and at 939, 919, 853 cm^{-1} (in modified starch) may be related to the band of higher saccharide derived from α-glucopyranose (930±9, 843±10, 761±8 cm^{-1})$^{9)}$. These bands may be related to the CC streching, CH_2 rocking vibrations.

(3) **OH group** The strong absorption in the region 3600～3000 cm^{-1} (cellulose), and the broad intense band near 3340cm^{-1} (polyvinyl alcohol) are due to the OH streching vibration. The strong absorption near 3330 cm^{-1} (in native starch), near 3350 cm^{-1} (in modified starch) may be related to the OH streching vibration. In both the starches, the exchangeable property for OH and OD under vapor phase was observed in this frequency region. The exchangeable ability or exchange rate seems to be larger in modified starch than in native starch. It may be dependent upon the crystalline structure, destruction or rearrangement of micell. The inference may be similar to the results from X-ray diffraction. The OD band has its corresponding OH band, with almost the same relative intensity, at the position displaced by a factor of 1.34～1.36 as shown in Table VI.

TABLE VI.

Specimen	OH bands obsd.cm^{-1}	OD bands obsd.cm^{-1}	Freq. ratios
native	3328	2468	1.35
modified	3350	2461	1.36

In the region 1550～1200 cm^{-1}, the CH deformation vibration may be associated with the OH deformation vibration. In the case of cellulose, a broad band with two peaks at 1446, 1335 cm^{-1} which were assigned to the OH in plane deformation was reported. Some$^{7)}$ of the vibrations of the two groups appear to interact each other. In the undeuterated material, the CH deformation vibration interacts with the OH deformation vibration. This may happen if the OH deformation vibration falls close to the CH deformation vibration, with resonance occurring. In such a case, of course, the bands are mixtures of these modes. Thus, on deuteration the OH deformation vibration is shifted too far from the CH deformation vibration. This explanation may be applied in this case roughly speaking.

(4) **CO and CC groups** The assignment of the bands relating to CO and CC streching vibration is more difficult than the hydrogen motions, because most probably there is some interaction between CO and CC streching vibrations. The band which did not appear in native starch appeared near 1700 cm^{-1} in modified starch, it remained unchanged on deuteration. Nitrogen could not determine by chemical analysis. Perhaps, the band may be related to CO. On the other hand, the band which did not appear in native starch appeared at 1589 cm^{-1} on deuteration in modified starch. This assignment will later be attempted. In the region 1200～850 cm^{-1}, there are many band which are as-

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cribed to the complicated vibrations including mostly the CO and CC streching vibrations. On the other hand, the CO streching vibration gives rise to an absorption band at 1034 cm^{-1} (methanol)(10), at 1070 and 885 cm^{-1} (methanol)(11) at 1096 cm^{-1} (polyvinyl alcohol). In the case of cellulose, the bands at 988, 965, and 895 cm^{-1} are ascribed to the CO, CC streching, CH_2 rocking vibrations. The strongest absorption peak appeared at 1003 cm^{-1} (in native starch) at 1021 cm^{-1} (in modified starch). The difference may be related to the physiochemical property (configuration etc.). The band at 851 cm^{-1} (polyvinyl alcohol), at 885 cm^{-1} (ethanol) is ascribed to the CC streching vibration. The band at 762 cm^{-1} (in native starch), at 760 cm^{-1} (in modified starch) may be related to the band of higher saccharide(9).

The description of the assignments of the bands did not repeat in each terms (1)–(4).

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Some Effects of Ethanolic Extracts of Potatoes on Phosphorylase Activity

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Effects of materials extracted from potatoes with hot 80% ethanol on the course of phosphorylase reactions were examined. These materials had no effect on phosphorylase activities as determined by the rate of liberation of inorganic phosphate from glucose-1-phosphate in the presence of a large amount of primer; however, in the presence of a small amount of primer or in its absence, these materials had significant effects on the liberation of inorganic phosphate and starch formation. These results suggested the presence of a small amount of primers in these materials. Evidence was presented against the participation of D-enzyme on the formation of starch when partially purified preparations of potato phosphorylase or crude extracts from potatoes were used as the enzyme.

Arreguin-Lozano and Bonner[1] have reported that a preparation obtained from 80% ethanolic extracts of potatoes which had been stored at 25°C contained a very potent phosphorylase-inhibitor. It was claimed that the formation of starch from G-1-P[*], as measured by the color intensity of the iodine-starch complexes, was seriously suppressed when E-E (H) was added to the reaction mixture containing partially purified potato phosphorylase and a small amount of added primer in addition to G-1-P; on the other hand, potatoes stored at 0°C did not yield such a phosphorylase-inhibitor.

Since then, the effects of E-E's on phosphorylase have been the subject of several investigators[2~4]. Porter and Rees[3] showed that E-E had a marked effect on the iodine-staining properties of the products of the phosphorylase reaction. However, they observed that E-E had a rather stimulating effect on the liberation of P*i* from G-1-P as catalyzed by phosphorylase. These findings could be interpreted quite reasonably from the following facts: (1) E-E contained glucose and amylodextrins of low molecular weights, the latter serving as natural primers in the phosphorylase reaction, and (2) the phosphorylase preparation used in their experiments, which had been partially purified by the method of Hanes[5], contained at least a factor, most probably the D-enzyme of Peat et al.[6], which modified the properties of products (amylose-type polysaccharides) of the phosphorylase reaction.

In view of the importance of such a phosphorylase-inhibitor in the determination of phosphorylase activities in crude extracts from plants as well as for obtaining a deeper insight to the carbohydrate metabolism of potatoes under different temperature conditions, the present author examined the effect of E-E on phosphorylase activity and showed that it had no inhibitory effect on phosphorylase as determined by the liberation of P*i* in the presence of a large amount of primer polysaccharide[2].

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[*] The following abbreviations are used throughout this paper: G-1-P, α-D-glucose-1-phosphate; ATP, adenosine triphosphate; *Pi*, inorganic orthophosphate; E-E, material obtained by extracting potatoes with hot 80% ethanol and removing ethanol by distillation in vacuo; E-E (H) and E-E (C), E-E obtained from potatoes stored at high (27°C) and low (3°C) temperatures, respectively.

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As the present author has not been successful in demonstrating the presence of D-enzyme in potatoes, it was intended to repeat the experiments of Porter and Rees in view of their evidence supporting its presence. The results of such experiments, which are given in this paper, show that (1) natural primers and glucose are present in E-E, in agreement with the results of earlier investigators, and (2) there is no detectable D-enzyme activity, at least under the conditions for the determination of phosphorylase activity, in partially purified preparations of potato phosphorylase or in crude extracts from potatoes. Moreover, the amount of natural primers present in E-E is presumably much smaller than that in a similar preparation of Porter and Rees; accordingly, together with the apparent absence of D-enzyme in phosphorylase preparations, the effects of E-E on phosphorylase reaction is very much different from the result obtained by Porter and Rees.

EXPERIMENTAL

Materials

G-1-P : Crystalline dipotassium salt of G-1-P was prepared according to the method described elsewhere[7].

ATP : Barium salt of ATP was prepared from rabbit muscle according to the method of Dounce et al.[8].

Soluble starch : It was prepared from commercial purified potato starch by the method of Lintner[9].

Achroic dextrin : Achroic dextrin, which was prepared from hydrol by the charcoal-Celite column chromatographic method of Whistler and Durso[10], was kindly supplied by Ass. Prof. H. Fukuba.

Amylose : It was prepared from potato starch according to the method of Schoch[11].

Partially purified potato phosphorylase : This was prepared by the method described elsewhere[12].

Potatoes : Potato tubers (*Solanum tuberosum* L. var. Irish cobbler) were stored either at 27±1.5°C or at 3 ±1.5°C for at least 2 weeks.

Crude extracts from potatoes : Peeled potatoes were ground with a porcelain grinder. The brei was pressed through linen cloth and the extracted juice was centrifuged at 7,000 r.p.m. for 5 min. The supernatant was stored in an ice-box for several days without significant loss in enzymic activity.

E-E : Peeled potatoes were grated into 4 vols. of hot 95% ethanol and the mixture was refluxed for 2 hrs. After cooling, the mixture was filtered through a Buchner funnel. The residue was washed with 1 vol. of hot 80% ethanol. The combined filtrate and washing were concentrated in vacuo to a small volume and then diluted to give a solution, 1 ml of which corresponding to 2 g. of fresh potatoes. Insoluble materials were removed by centrifugation, and the solution was stored in an ice-box.

Hexokinase : It was prepared from bakers' yeast according to the method of Slater[13]. The second ammonium sulfate fraction was dissolved in a small amount of water and stored frozen at −20°C. One ml of this solution phosphorylated 31.4 mg of glucose in 1 min. (pH 8.0; 37°C).

Glucose oxidase : De-oxin (glucose oxidase-catalase complex for analytical use), purchased from Nagase & Co., was used.

Methods

Determination of phosphorylase activity : The assay procedure has been described elsewhere[14], except 0.5 ml of 5% soluble starch solution was used as the added primer. Protein was determined by the biuret method of Gornall et al.[15].

Determination of reducing sugar : The colorimetric micro-method of Somogyi[16] was used.

Determination of starch-iodine complex formation : One-tenth ml of the sample solution was treated with 0.2 ml of 0.2% I_2–2% KI solution and the mixture was diluted to 10 ml with water. The optical density of the solution was measured in a 1-cm cuvette with a Hitachi model EPO-A photoelectric colorimeter fitted with R-filter (maximum absorption at 670 mμ) or with a Hirama model II photoelectric colorimeter fitted with a 610 mμ filter.

Paper chromatography of sugars : Tôyô No. 50 filter paper was used. The solvents used were BuOH-EtOH-H_2O (10 : 1 : 2)[17] and BuOH-pyridine-H_2O

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TABLE I. PHOSPHORYLASE ACTIVITIES OF CRUDE EXTRACTS FROM POTATOES STORED AT HIGH AND LOW TEMPERATURES FOR 16 DAYS

Temperature of Storage	Phosphorylase activity units/ml	Protein content mg/ml	Specific activity units/mg protein
27±1.5°C	6.37	15.32	0.42
3±1.5°C	8.78	14.04	0.63

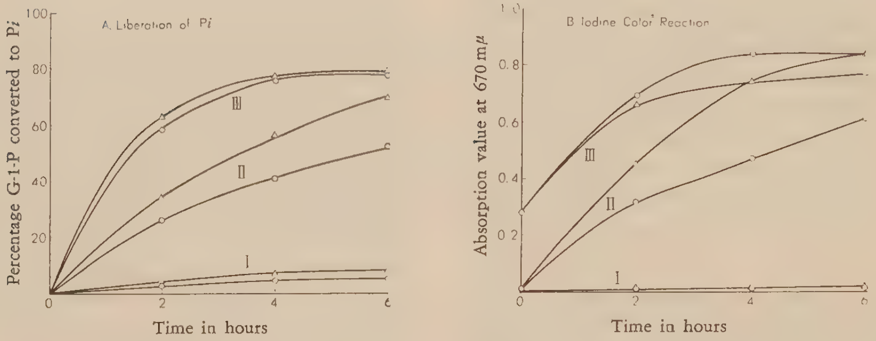

FIG. 1. Phosphorylase Activities of Crude Extracts of Potato Tubers Stored at 27°C or at 3°C for 27 Days.

Each reaction mixture contains, in a total volume of 3.5 ml, 0.143 M maleate buffer of pH 6.0, 0.0286 M G-1-P and 0.1 ml of crude potato extract. Other additions are: curves II and III, 0.7 mg and 17.5 mg of soluble starch, respectively. Primer was not added to curve I. ○——○, Extract of potatoes stored at 27°C, △——△, extract of potatoes stored at 3°C. Temperature, 37±0.1°C.

(6:4:3)[18]. The multiple (3 times) ascending method was employed. Sugars on the chromatogram were detected by aniline hydrogen phthalate[19] or bezidine-acetic acid-trichloroacetic acid[20] reagent.

RESULTS

Phosphorylase activities in crude extracts from potato tubers stored at high and low temperatures.

Although Arreguin-Lozano and Bonner reported that there was no difference between phosphorylase activities in crude extracts of potatoes stored at 0°C or at 25°C for 3 weeks, it was usually not the case in the author's experiments. For example, phosphorylase activities in potatoes stored at 27°C for 16～40 days were 62～95 % (av. of six experiments, 75 %) as high as those in potatoes stored at 3°C for the same periods. Protein contents in crude extracts were usally higher in the former than in the latter, and consequently, specific activities were lower in the former than in the latter. An example is given in Table I.

The lower phosphorylase activities in potatoes stored at a high temperature were not due to the presence of an inhibitor in these extracts, but to the lowered contents of phosphorylase itself, because it was confirmed that when these extracts or heat-inactivated extracts were added to a similar extract from potatoes stored at a low temperature, the former showed no inhibitory effects on phosphorylase activities of the latter.

Experiments in which the progress of the phosphorylase reaction was followed either by the liberation of Pi from G-1-P or by the starch-iodine complex formation by the action of these two extracts are shown in Fig. 1, A and B, respectively. In the absence of the added primer, there was very little liberation of Pi or starch-iodine complex formation; this showed that crude extracts of potatoes contained very little,

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[18] D. French and G. M. Wild, *J. Am. Chem. Soc.*, **75**, 2612 (1953).

[19] S. M. Partridge, *Nature*, **164**, 443 (1949).

[20] J.S.D. Bacon and J. Edelman, *Biochem. J.*, **48**, 114 (1951).

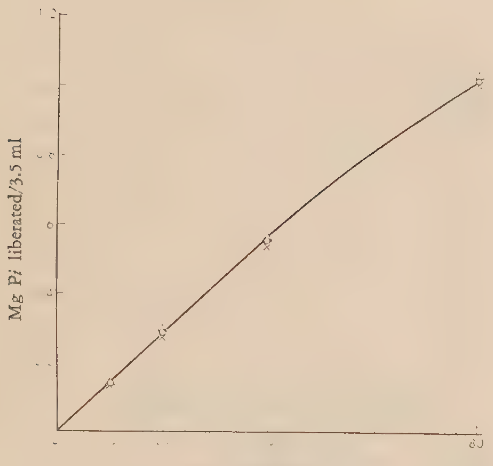

FIG. 2. Effects of E-E's on Phosphorylase Activities in the Presence of a Large Amount of Added Primer.

Each reaction mixture contains, in a total volume of 3.5 ml, 0.143 M maleate buffer of pH 6.0, 0.0286 M G-1-P, 0.714% soluble starch and 0.3 ml of partially purified potato phosphorylase (1.38 units/ml). Other additions are: △, 1 ml of E-E (H), ×, 1 ml of E-E (C). Temperature, 37±0.1°C.

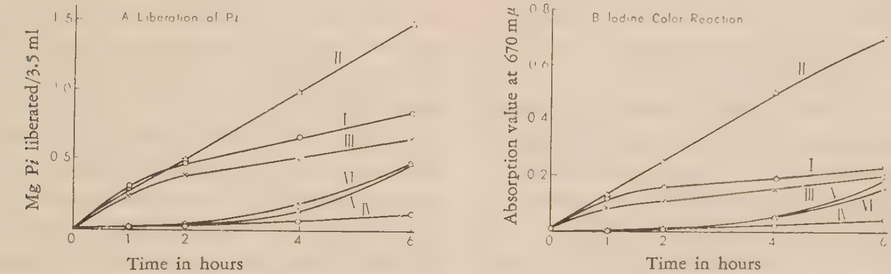

FIG. 3. Effects of E-E's on the Phosphorylase Activities in the Presence of a Small Amount of Primer or in the Absence of Primer.

Each reaction mixture contains, in a total volume of 3.5 ml, 0.143 M of maleate buffer of pH 6.0, 0.0286 M of G-1-P and 0.3 ml of partially purified phosphorylase. Other additions are: curves I-III, 0.7 mg of soluble starch; curves II and V, 1 ml of E-E (H); and curves III and VI, 1 ml of E-E (C). Temperature, 37±0.1°C.

if not at all, priming oligo- or polysaccharides.

Effect of E-E on phosphorylase activity.

Effects of added E-E's on partially purified potato phosphorylase in the presence of a large amount of primer are shown in Fig. 2. As it is clear from the figure, E-E's in amount corresponding to 2 g of fresh potatoes per 3.5 ml reaction mixture had no inhibitory effects on phosphorylase activities. Experiments in which a small amount of primer was either added or not added at all are shown in Fig. 3. From the figure (see curve IV) it is evident that there are practically no primer contaminants present in the phosphorylase and G-1-P preparations used in these experiments. When E-E was added to the phosphorylase reaction system in the absence of added primer, there was a slight liberation of Pi and starch formation, indicating the presence of a small amount of primer in E-E. However, in view of the long lag period in the absence of added primer, the amounts might indeed be very small. Qualitative paper chromatography and quantitative ion-exchange chromatography[21] of these E-E's showed that they contained large amounts of sucrose and small amonnts of glucose and fructose; the contents of oligosaccharides were so small that it was impossible to detect them by these methods. There was no significant difference in the contents of primers between E-E's prepared from potatoes stored at high and low temperatures. In the presence of a small amount of added primer, E-E (H) had a marked activating effect on the liberation of Pi and starch formation, while E-E (C) had, on the contrary, a slight retarding effect; this is just contrary to the results of Arreguin-Lozano and Bonner. Such a marked difference in the effects of E-E's

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21) K. Mori and M. Nakamura, This Bulletin, **23**, 389 (1959).

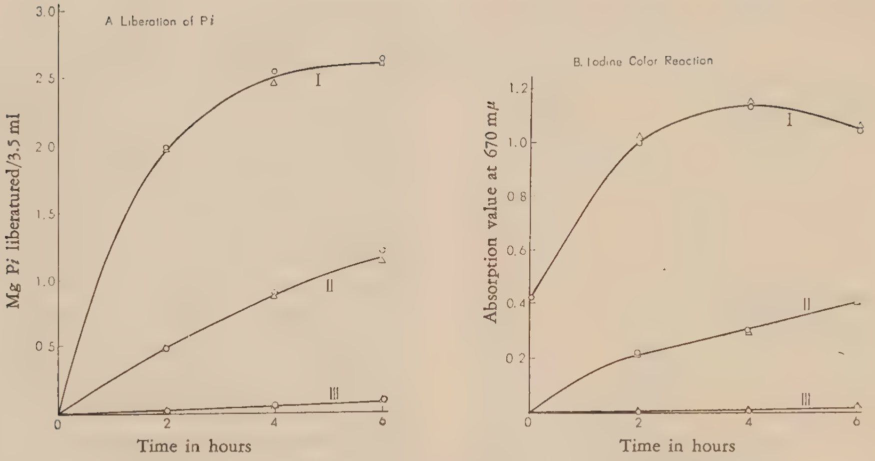

FIG. 4. Effect of Added Glucose on Partially Purified Phosphorylase.

Each reaction mixture contains, in a total volume of 3.5 ml, 0.143 M of maleate buffer of pH 6.0, 0.0286 M of G-1-P and 0.3 ml of partially purified phosphorylase. Other additions are: curve I, 17.5 mg of soluble starch; curve II, 0.7 mg of soluble starch; ○, no glucose; △, 7mg of glucose. Temperature, 37±0.1°C.

in either the presence or absence of added primer may be due to the difference in the quality and quantity of primer oligosaccharides contained in these E-E's. At the present time it is hard to give a clear-out interpretation in regard to these phenomena.

In similar experiments of Porter and Rees in the presence of a small amount of added primer, the reaction product formed stained blue with iodine at an early stage, but as reaction proceeded, the intensity of the blue color was reduced. Such a reversion of the iodine color reaction did not occur in the author's experiments.

Effect of added glucose

The experiments of Porter and Rees suggested the presence of D-enzyme in their partially purified phosphorylase preparation on the ground that the addition of E-E or glucose to the phosphorylase reaction mixture in the presence of a small amount of added primer caused reversion of the iodine color reaction during the course of enzymic action and that C^{14}-labeled glucose served as the co-substrate in the reaction forming C^{14}-labeled oligosaccharides.

As shown in the preceding section, no reversion of iodine color was observed in the author's experiments. Further evidence against the presence of D-enzyme is given in Fig. 4, in which the possible presence of D-enzyme was tested by using glucose as the co-substrate in place of E-E. The addition of glucose had no effect on the course of phosphorylase reaction when the reaction was followed either by the liberation of Pi or by iodine color reaction. Crude extracts of potatoes were also used as the enzyme source to test the possibility that D-enzyme might have been removed during the purification procedures. Again, there was no indication for the presence of D-enzyme, as shown in Fig. 5.

Effect of the addition of achroic dextrin.

Fig 5 shows experiments in which various amounts of achroic dextrin were added as the primer. When a large amount of dextrin was added to the reaction mixture, the rate of liberation of Pi was rapid, while the iodine coloration of the products turned via brown to wine-red and not to blue. On the contrary, when a small amount of dextrin was added, the rate of liberation of Pi was slow, but the products showed a blue iodine coloration. The difference in these two cases may be attributed to the difference between the ratio of the amount of primer to

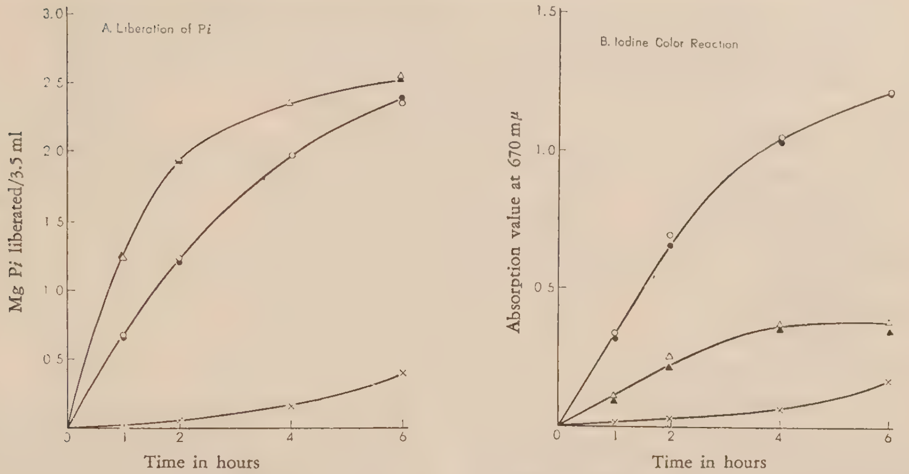

FIG. 5. Effect of Added Glucose on Phosphorylase Activity in the Presence of Achroic Dextrin.

Each reaction mixture contains, in a total volume of 3.5 ml, 0.143 M of maleate buffer of pH 6.0, 0.0286 M G-1-P and 0.1ml extract of potatoes stored at 27° for 7 weeks. Other additions are: circles, 0.7 mg of achroic dextrin; triangles, 10 mg of achroic dextrin; open signs, without added glucose; closed signs, 7 mg of glucose. Temperature, 37±0.1°C.

G-1-P. Addition of glucose to these reaction mixtures has no effect on the liberation of *Pi* and iodine coloration.

Direct evidence for the apparent absence of D-enzyme in potato extracts.

Although the results obtained above suggested the absence of D-enzyme in the partially purified phosphorylase preparations or in crude potato extracts, a more direct evidence for the absence of D-enzyme was obtained by using achroic dextrin or amylose as the substrate in the presence and absence of glucose. The results obtained by using partially purified phosphorylase are shown in Table II. If D-enzyme is acting in the reaction mixture containing achroic dextrin in the absence of added glucose, it is to be anticipated that a product which is stained blue with iodine may be formed. The addition of glucose to this system will cause inhibition of the formation of amylose-like polysaccharide and, at the same time, the reducing power will be diminished. If amylose is used as the substrate in the presence of glucose, it will result in reductions in the intensity of blue color and reducing power. However, this was not the case as it is shown in Table II; when partially purified phosphorylase or crude potato extract was used as the enzyme, there was practically no change in the reducing power or in the iodine coloration of these reaction mixtures. When amylose was used as the substrate, the reduction in the iodine stain was not greater than that observed as a consequence of spontaneous retrogradation of amylose.

In other experiments, a glucose oxidase-catalase system or a hexokinase-ATP system was added to the enzymic mixture to remove any glucose formed from achroic dextrin by the action of D-enzyme. This treatment will shift the equilibrium of the D-enzyme reaction toward the formation of higher oligosaccarides and, consequently, facilitate the detection of iodine-stainable polysaccharides. The reaction mixture (5 ml) contained 0.05 M maleate buffer of pH 6.1, 1.5 % of achroic dextrin, 0.2 ml of potato extract and either (1) 0.1 % Deoxin (30 units/ml) or (2) 0.1 ml of yeast hoxokinase, 0.01 M ATP, 0.01 M $MgCl_2$ and 0.01 M NaF. Also in these experiments, the formation of iodine-stainable polysaccharides or change in reducing power could not be observed.

TABLE II. ABSENCE OF D-ENZYME IN PARTIALLY PURIFIED POTATO PHOSPHORYLASE.

Each reaction mixture contains 0.3 ml of 0.5 M maleate buffer of pH 6.0 and 0.3 ml of partially purified potato phosphorylase (2.76 units/ml) in addition to those given in the table. Total volume, 2.1 ml. Temperature 37±0.1°C.

Expt. no.	Additions	Absorption value at 610 mμ					Reducing power*				
		0 hr.	1.5 hr.	3 hr.	5 hr.	7 hr.	0 hr.	1.5 hr.	3 hr.	5 hr.	7 hr.
1.	18 mg of achroic dextrin	0.001	0.004	0.007	0.007	0.005	2.62	2.51	2.78	2.64	2.87
2.	18 mg of achroic dextrin +4.6 mg of glucose	0.004	0.003	0.005	0.005	0.005	8.02	9.30	8.97	9.16	10.01
3.	9 mg of amylose	1.00	0.92	0.94	0.88	0.87	0.45	0.38	0.17	0.13	0.34
4.	9 mg of amylose +4.6 mg of glucose	0.95	0.94	0.93	0.89	0.87	7.10	6.68	6.80	6.80	6.52
5.	9 mg of amylose (enzyme not added)	0.98	0.92	0.90	0.88	0.86	0.60	0.35	0.16	0.16	0.24

* Calculated as mg glucose.

DISCUSSION

The results obtained above are in agreement with those of Porter and Rees in that there is no phosphorylase-inhibitor in E-E and that E-E contains natural primers for phosphorylase. However, there are some discrepancies between these two results, some of which may be enumerated as follows:

(1) The amount of natural primers contained in the author's E-E was far lower than that of Porter and Rees. This is evident from a comparison of the results shown in Fig. 3 of this paper and those (see Figs. 2 and 3) obtained by Porter and Rees. However, as the phosphorylase system of Porter and Rees contained a rather large amount of primer in the absence of added soluble starch (see Fig. 1), it is impossible to make a quantitative comparison on this point.

(2) The partially purified phosphorylase or crude extracts of potatoes used in the author's experiments indicated the absence of D-enzyme in these solutions. This is in a sharp contrast with the results of Porter and Rees, who clearly indicated the presence of D-enzyme in their partially purified phosphorylase preparation. The presence of D-enzyme in potatoes has been established by several investigators[6] including Walker and Whelan[22], who have clearly shown the formation of iodine-stainable polysaccharides from maltotetraose in the presence of the hexokinase-ATP system. It is not clear whether the difference in the varieties of potatoes or the climatic conditions for the cultivation of potatoes might be attributed to the failure of the present author to demonstrate D-enzyme activity in his potatoes.

Schwimmer and Weston[23] have reported that P*i* contained in E-E has no effect on the liberation of P*i* from G-1-P as catalyzed by phosphorylase, but that it inhibits the formation of amylose. Schwimmer has also found that E-E contains natural primers[4] and moreover, a true inhibitor of phosphorylase which is identified as chlorogenic acid[24, 25]. According to Schwimmer, quinic acid, caffeic acid and epinephrine are also potent inhibitors of phosphorylase. However, as the inhibitory effects of chlorogenic acid is evident only after the primers are removed from E-E, it will not have effect on the determination of phosphorylase activities which are usually carried out in the presence of a large amount of added soluble starch.

Acknowledgment The author wishes to express his sincere thanks to Prof. S. Funahashi for his interest in this work and to Ass. Prof. H. Fukuba, Ochanomizu University, for his generous gift of achroic dextrin.

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Studies on Transfer of Antiseptics to Microbes and their Toxic Effect

Part I. Accumulation of Acid Antiseptics in Yeast Cells

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In the medium of the low pH value, acid antiseptics (salicylic, benzoic, dehydroacetic and sorbic acid) transfer very rapidly from the medium to yeast cells, and these antiseptics are accumulated in the cells.

The transferred quantity is in equilibrium with the concentration of the undissociated antiseptics in the medium, and the ratio of the transferred quantity to the concentration of the total antiseptics in the medium is limited by the pH in the medium. Accordingly, it has been considered that the pH value affects the transfer ratio and results in the variation of the toxic effect of the antiseptics.

In connection with this, not only the ionization equilibrium, but also the adsorption of the undissociated antiseptics on the solid phase of the cell has been assumed to be related to the accumlatian of the antiseptics.

In order that antiseptics inhibit the growth of microbes, it is essential to transfer them from a medium to microbe cells, and thus the toxic effect will be influenced by the quantity of the antiseptics transferred to the cells.

Usually, the toxic effect of antiseptics on microbes in food preservation is estimated by the concentration required to bring about a certain response. However, no information can be obtained by this method on the net toxic activity because the quantity transferred to the microbes remained unknown.

It is well known that the toxic effect of acid antiseptics such as salicylic, benzoic, dehydroacetic and sorbic acid, which have an ionization constant in the order of $10^{-3} \sim 10^{-5}$, varies with the hydrogen ion concentration of the medium, and that the toxic effect is proportional to the concentration of the undissociated antiseptics. Accordingly, it has been assumed that the undissociated fraction of acid antiseptics is the chief substance, and in many cases the only substance having a relation to the antiseptic effect[1~4].

Recently, Masuo and Okabayashi[5] have been suggested that only the undissociated molecules of dehydroacetic acid exclusively permeate into microbe cells and that the concentration of the undissociated molecules is proportional to the toxic effect. The same mechanism was supported by Nomoto et al.[6] in connection with the toxic effect of sorbic acid.

Conway and Downy[7] have obtained an interesting result that only the undissociated molecules of acetic acid selectively diffuse into yeast cells, and that the diffused acid is accumulated in the inner cell fluid in the ionized form. It seems that this mechanism can also be applied

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[1] P. G. F. Vermast, *Biochem. Z.*, **1921**, 125.

[2] S. Tetsumoto, *J. Agr. Chem. Soc. Japan*, **9**, 388, 563, 761, 1284 (1933).

[3] O. Rahn and J. E. Conn, *Ind. Eng. Chem., Ind. Ed.*, **36**, 185 (1944).

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to the transfer of acid antiseptics, though the mechanism of the selective diffusion is a matter of speculation.

As mentioned above, only a little information has been obtained on the transfer mechanism of acid antiseptics and on the relation between the transfer and their toxic effect.

As the result of the present work, it has been considered that the pH value limits the ratio of the transferred quantity to the concentration of the total antiseptics in the medium and results in the variation of the toxic effect of the antiseptics, and that not only the ionization equilibrium, but also the adsorption of the undissociated antiseptics on the solid phase of the cell is related to the accumulation of antiseptics.

MATERIALS AND METHODS

Yeast cells Bakers' yeast was repeatedly washed with tap water and centrifuged for twenty minutes at a rate of 4,000 rotations per minute. The quantity of water which wetted the surface of the centrifuged yeast was estimated by Conway's inulin space method[8]. The constitution of the centrifuged yeast is shown in Table I.

TABLE I. CONSTITUTION OF CENTRIFUGED YEAST (%)

Cells	78.3
Dry matter	27.1
Water in cells	51.2
Wetting water out of cells	21.7

Acid antiseptics Salicylic acid (m.p. 157°~159°), benzoic acid (m.p. 122°), dehydroacetic acid (m.p. 108°) and sorbic acid (m.p. 133°) were used in the form of the sodium salt.

Buffer solution McIlvaine's buffer solution was used, here the ionic strength of the solution was adjusted to 0.42 with sodium chloride.

Determination of transferred quantity A definite quantity of the centrifuged yeast was suspended in the buffer solution, and a definite quantity of an antiseptic and distilled water was added to the suspension. Finally, the buffer solution was diluted to a quarter of the original concentration. The quantity of the yeast cells in the final mixture was 1 per cent in the case of salicylic acid, and was 5 per cent in the cases of other antiseptics. The temperature of the suspension was held constant. After definite intervals, the mixture was taken out and centrifuged. Then, the concentration of the antiseptic in the supernatant was determined and its pH value was measured. Simultaneously, a blank experiment was carried out under the same condition.

The "transferred quantity", a (m-mol./kg-cell), of the antiseptic was calculated by equation (1). Here, C_o and C_m (m-mol./kg-water)

$$a=(C_o-C_m)V/M \tag{1}$$

are the concentrations of an antiseptic in the medium before and after suspending the yeast, respectively, and V(kg), the final quantity of the solvent water in the medium, and M (kg), the weight of the suspended yeast cells.

The "transfer ratio", A (kg-water/kg-cell), was calculated by equation (2).

$$A=a/C_m \tag{2}$$

Determination of antiseptics in medium The supernatant of the mixture was diluted with 0.2 per cent aqueous hydrochloric acid, and the optical extinction of this solution was measured by Shimizu ultraviolet-spectrophotometer. The blank test was also carried out in the same manner. Then, the exact extinction of the antiseptic in the solution was given by the difference of both values.

Since the extinction value of the antiseptics used in this experiment was proportional to the concentration in this condition, the concentration of the antiseptics in the supernant was calculated by their molar extinction coefficient and the dilution ratio of the supernatant. Wave-length and molar extinction coefficient are given in Table II.

Measurement of pH value The pH value of the medium was measured by Tōyō Roshi glass-electrode pH meter.

TABLE II. OPTICAL ABSORPTION DATA OF ACID ANTISEPTICS IN 0.2 PERCENT AQEOUS HYDROCHLORIC ACID SOLUTION

Acid antiseptic	Wavelength at maximum absorption (mμ)	Molar extinction coefficient
Salicylic acid	236.5	8,370
Benzoic acid	230.0	11,580
Dehydroacetic acid	307.0	13,060
Sorbic acid	260.0	25,400

RESULTS

Change of transfer ratio with suspending time Changes of the transfer ratio with suspending time were subsequently investigated. The re-

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8) E. J. Conway and M. Downy, *Biochem. J.*, **47**, 347 (1950).

TABLE III. CHANGE OF TRANSFER RATIO WITH SUSPENDING TIME

	Transfer ratio					
Suspending time (min.)	5	10	15	30	60	120
Dehydroacetic acid	7.50	7.50	7.34	7.42	7.34	7.44
Sorbic acid	16.5	17.0	17.5	17.9	17.5	18.3
Benzoic acid	31.7	36.4	35.9	36.8	34.2	35.5
Salicylic acid	72.1	72.5	70.4	72.2	72.9	71.2

Yeast cells were suspended in the buffer solution of pH 3.20 containing of an acid antiseptic in 1 m-mol./kg-water at 30°C.

sults are shown in Table III. The transfer of the antiseptics seems to be practically completed in less than five minutes at pH 3.20.

Influence of pH on transfer The transfer ratio changed according to the pH value in the medium as shown in Fig. 1. On the other hand, the ratio of the undissociated fraction to the total quantity of the corresponding antiseptic in the medium was calculated from the ionization constant, and is appended in Fig. 1. It is note-

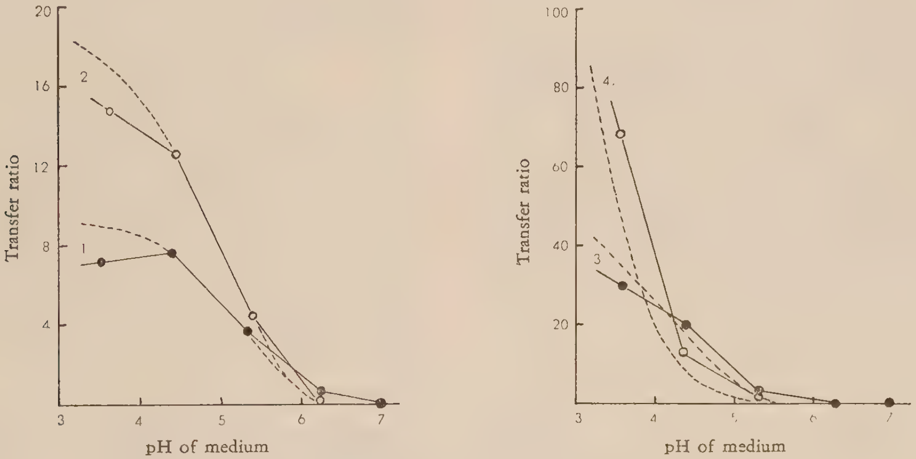

FIG. 1. Change of Transfer Ratio of Acid Antiseptics by pH of Medium

1: Dehydroacetic acid (pK 5.1) 2: Sorbic acid (pK 4.8)
3: Benzoic acid (pK 4.2) 4: Salicylic acid (pK 3.0)

Yeast cells were suspended in the buffer solution containing an acid antiseptic in 1 m-mol./kg-water for one hour at 30°C. Actual lines show the experimental data. Dotted lines show the theoretical ratio of the undissociated fraction of the antiseptics. Latters were enlarged to cross on the pH point of the corresponding experimental courves.

TABLE IV. INFLUENCE OF pH OF MEDIUM ON TRANSFER AND REMOVEMENT OF ACID ANTISEPTICS

	Quantity of antiseptic in medium (μ mol.)			
Antiseptic	Dehydroacetic acid	Sorbic acid	Benzoic acid	Salicylic acid
Antiseptic added	20.0	20.0	20.0	20.0
Residual at pH 3.15[1]	15.3	12.6	8.7	14.2
Residual at pH 7.02[2]	19.8	19.9	19.5	19.5
In neutralized medium[3]	20.1	19.8	19.3	20.3
pH of neutralized medium	6.83	6.96	7.01	7.15

Yeast cells were suspended into 10 ml of the medium containing 2 μmol. of an acid antiseptic at pH 3.15 or 7.02. After one hour, the residual quantity of the antiseptic[1][2] was determined.

The suspension at pH 3.15 was neutralized with sodium hydroxide to pH 6.8~7.2 and was allowed to stand for one hour at 30°C. Then, the quantity of the antiseptic in the medium[3] was determined.

worthy that the shape of the observed curves seems to coincide with that of the calculated curves. From this result, it can be concluded that the transferred quantity is in equilibrium with the concentration of the undissociated antiseptics in the medium and the transfer ratio is thus limited by the pH in the medium.

At the low pH value of the medium a large amount of the antiseptics is transferred to the cells. When the medium was neutralized and was allowed to stand for one hour, the antiseptics in the cells were easily and perfectly removed as shown in Table IV. This result indicates that no antiseptics decomposed, and that the transfer equilibrium is controlled by the pH value of the medium.

Change of transfer ratio by equilibrium concentration The relation of the equilibrium concentration in the medium with the transferred quantity is shown in Table V.

From the result shown in Table V, it is seen that the transferred is not linearly proportional to the equilibrium concentraiton, and that the transfer ratio is reduced as the equilibrium concentration is increased.

The plot of the logarithm of the transferred quantity versus the logarithm of the equilibrium concentration gives a straight line as shown in Fig. 2. This relation can be expressed by equation (3),

$$a=k(C_m)^n \qquad (3)$$

where k and n are the constants concerning each antiseptic, a the transferred quantity, and C_m the equilibrium concentration of an antiseptic. It is of interest that equation (3) corresponds to Freundlich's adsorption isotherm.

Dependence of transfer on temperature The relation of the transferred quantity to the equilibrium concentration was examined at various temperatures, and the values of k and n at various temperatures are given in Table VI. These values seem to vary with the temperature.

DISCUSSION

In the medium with the low pH value, the acid antiseptics transfer very rapidly from the medium into the yeast cell, and the antiseptics are accumulated in the cell (Tables III and V). The transferred quantity is in equilibrium with the concentration of the undissociated antiseptics in the medium, and therefore the transfer ratio of the antiseptics is limited by the pH in the

TABLE V. CHANGE OF TRANSFER RATIO BY EQUILIBRIUM CONCENTRATION

Antiseptic		Initial concentration (m-mol./kg-water)	Equilibrium concentration (m-mol./kg-water)	Transferred quantity (m-mol./kg-cell)	Transfer ratio
Dehydroacetic acid	(cells : 5%)	0.25	0.168	1.64	9.76
		0.50	0.350	3.00	8.57
		1.00	0.729	5.42	7.43
		2.00	1.528	9.44	6.17
Sorbic acid	(cells : 5%)	0.25	0.106	2.88	27.2
		0.50	0.239	5.22	21.8
		1.00	0.729	9.32	17.3
		2.00	1.258	14.84	11.8
Benzoic acid	(cells : 5%)	0.25	0.060	3.80	63.3
		0.50	0.169	6.62	39.1
		1.00	0.357	12.9	36.1
		2.00	0.874	22.5	25.7
Salicylic acid	(cells : 1%)	0.25	0.107	14.3	133.5
		0.50	0.244	25.6	104.8
		1.00	0.579	42.1	72.8
		2.00	1.370	63.0	46.0

Yeast cells were suspended in the buffer solution of pH 3.20 containing an acid antiseptic for one hour at 30°C.

TABLE VI. DEPENDENCE OF TRANSFER OF ACID ANTISEPTICS ON TEMPERATURE

	k			n		
Temperature (°C)	30	20	10	30	20	10
Dehydroacetic acid	6.9	10.0	8.3	0.78	0.76	0.70
Sorbic acid	13	16	14	0.68	0.65	0.53
Benzoic acid	25	27	25	0.68	0.54	0.46
Salicylic acid	56	51	59	0.61	0.60	0.57

The values of k and n correspond with the constants in equation (3). The experimental conditions were the same as is shown in Table V except the temperature of the suspension.

medium (Fig. 1). On the other hand, it has well been known that the toxic effect of the antiseptics is proportional to the ratio of the undissociated fraction to the total quantity of the antiseptics in the medium[1~6]. Accordingly, it is considered that the pH value affects the transfer ratio and results in the variation of the toxic effect of the antiseptics.

With respect to the case that only the undissociated acid is related to the transfer to the cell, Conway and Downy[7] have proposed equation (4). (In order to simplify problem, the correction for ionic strength was omitted from the original formula.)

$$R=C_lS/C_m=S(1+K/h_l)/(1+K/h_m) \quad (4)$$

Here, C_l and C_m are the concentrations of the acid in and out of the cell, K the ionization constant and S the quantity of the solvent water in the cell which was evaluated as great as 0.7 (l/kg-cell)[7]. In that case, the pH value in the cell is lowered by accumulation of the acid, and the decrease in the pH value is evaluated and corrected by the average buffering of the cell fluid (63 m-equiv./l-fluid)[7]. Thus, the pH value in the resting bakers' yeast cell has been evaluated to be 5.81 from the data with acetic acid and is in good agreement with the value obtained from direct determination of the pH value of frozen and thawed yeast[7].

Supposing that the value of C_lS in equation (4) corresponds to the transferred quantity, a, in the present experiment, the value of R will correspond to the transfer ratio, A. Thus, this mechanism can be applied to the transfer of acid antiseptics, and the change of the transfer ratio of the antiseptics with pH in the medium (Fig. 1) and the trend that the smaller is the value of pK of the antiseptics, the greater is the maximum value of the transfer ratio (Fig. 1 and Table V) can be expressed by equation (4).

On the other hand, the relation between the transferred quantity and the equilibrium concentration obeyed Freundlich's adsorption isotherm (Fig. 2), and the values of k and n in the isotherm were varied with temperature (Table VI). From these facts, it may be assumed that the adsorption has a relation to the transfer and accumulation. However, the fact that the value of n in the isotherm is smaller

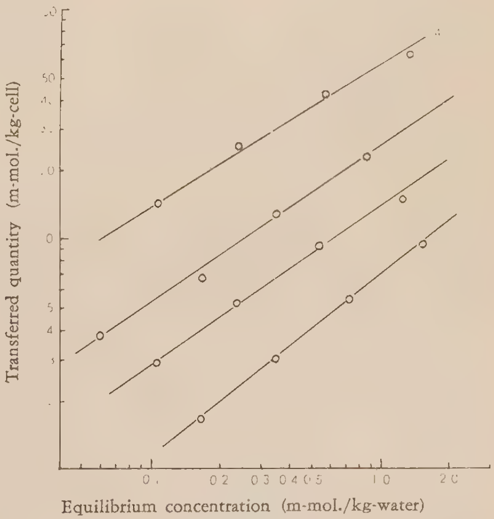

FIG. 2. Relation between Transferred Quantity and Equilibrium Concentration of Acid Antiseptics (pH 3.20)

1: Dehydroacetic acid
2: Sorbic acid
3: Benzoic acid
4: Salicylic acid

than 'one' is considered to depend partially upon the decrease in the pH in the cell owing to the accumulation of the antiseptics. Also, the influence of temperature on the values of k and n in the isotherm may be elucidated from the fact that the pH in the cell is influenced by the temperature. This is because the ionization constant of the antiseptics changes with temperature.

As mentioned above, equation (4) is apparently applicable to the present data. Then, the original pH value in the cell was evaluated by substituting the data given in Table V and Conway's S value (0.7) in equation (4) and by correcting the pH value which was decreased by the accumulation of the antiseptics.

TABLE VII. VALUE OF pH AND QUANTITY OF SOLVENT WATER IN YEAST CELL CALCULATED BY IONIZATION EQUILIBRIUM OF ACID ANTISEPTICS IN AND OUT OF CELL

Antiseptic		C_m[1] (m-mol./kg-water)	A[1]	pH (calc.)	pH[2] (original)	S-value[3]
Dehydroacetic acid	(pK 5.1)	0.168	9.76	6.23	6.26	1.7
		1.528	6.17	6.00	6.19	1.3
Sorbic acid	(pK 4.8)	0.106	27.2	6.29	6.35	2.2
		1.258	11.8	5.91	6.23	1.3
Benzoic acid	(pK 4.2)	0.060	63.3	6.19	6.28	1.7
		0.874	25.7	5.79	6.30	1.3
Salicylic acid	(pK 3.0)	0.107	133.5	5.63	5.95	0.92
		1.370	46.0	5.23	6.66	1.2
Acetic acid (author)		5.50	6.09	5.42	5.84	0.73
Acetic acid (Conway)	(pK 4.7)			5.44	5.81	0.70

1) These values were quoted from Table V.
2) On the assumption that the value of S is constant (0.7), the original pH in the cell was calculated by equation (4).
3) On the assumption that the original pH is constant (5.81), the value of S was calculated by equation (4).

However, the evaluated pH values in the cell given in Table VII are greater than those obtained with acetic acid, though they were expected to be equal (5.81). From the assumption that the pH value of yeast is equal to that of Conway's yeast (5.81), the value of S given in the last column of Table VII exceeds '0.7'. But, this evaluated value is fabulously great, because the maximum value of S should be smaller than '0.7', judging from the content of water in the yeast cell (Table I).

From this fact, it seems that not only the ionization equilibrium in the aqueous phase, but also the other possible mechanism such as adsorption or partition has a relation to the accumulation of the antiseptics. Supposing that the adsorption on the cell membrane and dissolution in the lipid of the cell are related to the selective transfer of the undissociated antiseptics, the fact that antiseptics transfer very rapidly to the cell (Table III) will be easily elucidated, while it cannot be elucidated by mere diffusion through the cell membrane. (Sugars and polyalcohols which have the same order of molecular weight as the present antiseptics hardly diffuse into the cell.) As the content of the lipid in the yeast usually does not exceed a low percentage, the quantity of the antiseptics remaining in the lipid phase by partition may be negligibly small, and thus the adsorbed quantity on the solid phase of the cell becomes important matter.

On basis of these considerations, it is most reasonable to assume that the main part of the transferred antiseptic exists in two states: one is the dissolved antiseptic in the inner cell fluid, which is in an ionization equilibrium, which is a function of pH, as proposed by Conway and Downy[7], and the other is the adsorbed antiseptic on the solid phase of the cell.

Investigation of the role of the dissolved and adsorbed antiseptic on the toxic effect will be presented in the following report.

Acknowledgements The author wishes to express his sincere thanks to Emeritus Prof. R. Sasaki and Prof. Y. Sakurai of Tokyo University for their interest in his work, and also to Dr. C. Hata, President of this Institute, for his support and encouragement. The author is greatly indebted to Dr. M. Fujimaki, Dr. S. Okimasu, Mr. R. Nōmi and Mr. K. Shimizu for their kind advice.

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Wood Extractives

Part IX. A New Diterpene Alcohol from *Cryptomeria japonica* D. Don

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Phyllocladanol, a saturated tetracyclic diterpene alcohol present in the neutral portion of methanol extract from *Cryptomeria japonica*, is shown to readily dehydrate to phyllocladene, and the constitution (I) is proposed. Occurrence of a further compound, isophyllocladene, in steam-volatile material of the extract is described.

In continuation of earlier work1) an unknown compound, $C_{20}H_{34}O$, m. p. 182~183°, $[\alpha]_D^{15}+14.52°$, obtained from the neutral portion of wood resin of *Cryptomeria japonica* D. Don, has been examined. In part VIII1) of this series, an isolation of this compound has been described in detail. This compound, called phyllocladanol, is not apparent to have been previously isolated. Phyllocladanol is colorless needles being soluble in ether and chloroform, less soluble in methanol, and give a negative Liebermann-Burchard reaction. In view of this molecular formula and co-existence in wood with other diterpenoids, e.g. ferruginol and xanthoperol, it is probable that phyllocladanol is one of diterpenes having a polycyclic carbon skeleton.

Phyllocladanol contains one hydroxyl group which is unreactive, not being acylated under ordinary conditions. It exhibits strong infrared absorptions at 3436 and 1126 cm^{-1} which are assigned to the hydroxyl group. Treatment with acetic anhydride an anhydrous sodium acetate under reflux unexpectedly gives a compound, m. p. 91~93°, corresponding to a mixture of monoacetyl derivative and its dehydration product. Having attempted mild oxidation with chromic acid the compound is recovered unchanged, in a small quantity. The hydroxyl group is accordingly considered to be tertiary.

In reference to the nature of carbon skeleton of the diterpene alcohol it is considered to have a tetracyclic structure as mentioned below. From the negative tetranitromethane reaction, no absorption of hydrogen on catalytic hydrogenation using platinum oxide catalyst in a glacial acetic acid solution, and the absence of a characteristic infrared absorption band for the double bond in the 1700~1600 cm^{-1} region, it is shown that phyllocladanol is a saturated compound. In view of the molecular formula and

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1) T. Kondo, H. Imamura and M. Suda, This Bulletin, **23**, 233 (1959).

presence of a tertial hydroxyl group, phyllocladanol must be tetracyclic. This is confirmed by the following way. Dehydration of the compound with phosphoryl chloride in pyridine gave a homogeneous product of which the infrared spectrum indicates the presence of a disubstituted ethylenic linkage (bands at 867, 1653 and 3058 cm^{-1}). These figures and a few additional features in infrared absorption band agreed with those reported by E. Mosettig and W.R. Nes[2] for a tetracyclic diterpene, phyllocladene. The dehydration product is identified as phyllocladene (II), $C_{20}H_{32}$, m.p. 98°, $[\alpha]_D^{15}+16.6°$, which has been isolated from numerous essential oils[3], by comparison of the physical constants with those cited in the literature[3], and by isomerization to isophyllocladene (III), m.p. 108°, $[\alpha]_D^{13}+22.2°$. On action of mineral acid the dehydration product smoothly converts into an isomer, isophyllocladene in a quantitative yield. Isophyllocladene exhibits distinct infrared absorption bands at 826, 1634 and 3058 cm^{-1} indicating the presence of a trisubstituted double bond as suggested by C.W. Brandt[4]. When phyllocladanol is treated with boiling ethanolic sulfuric acid solution or with potassium hydrogensulfate at 190°, dehydration effectively occurs affording the same anhydro-compound, isophyllocladene.

Identification of the dehydration compound as isophyllocladene is made through mixed melting point determinations of its derivatives, e.g. α-dihydrophyllocladene, $C_{20}H_{34}$, m.p. 72~74° and dibromide, $C_{20}H_{32}Br_2$, m.p. 150~151° (decomp.), with corresponding derivatives of a specimen which was immediately isolated from a higher boiling fraction of steam distillate of original wood extract, as mentioned below.

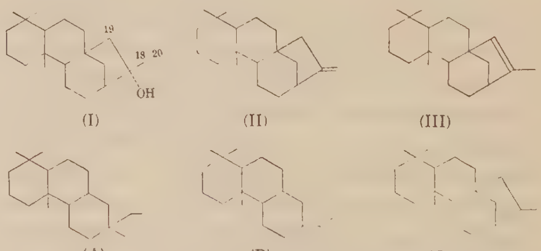
Evidence now available indicates that phyllocladanol must be expressed as the structure (I).

The general stereochemical feature of phyllocladanol remains undetermined. However, an analogy of the chemical behaviours of the diterpene alcohol, especially in its dehydration reaction and isomerization of anhydro product, to that of labdanolic acid[5] suggests that the C(18)*-hydroxyl group has the same equatorial configuration as one of the C(6)-hydroxyl group of the diterpene hydroxy acid.

Since phyllocladanol is isolated from the neutral steam-nonvolatile portion of the wood extract, an attempt was made to obtain further diterpenes from the steam-volatile material. After separation of the lower boiling portion (100~160°/0.3 mm. bath temp.) by fractional vacuum distillation, steam-distillate afforded an additional diterpene, isophyllocladene, m.p. 108° in a crystalline state, which has been previously shown to be present in the essential oil of *Phyllocladus trichomanoides* by L.H. Briggs et al.[7].

It is worth emphasising in contrast to T.G. Halsall's findings[8], that in nature, triterpenes (or steroids) and diterpenes do not appear to be found together, and the results with extracts of *C. japonica* show the co-existence of β-sitosterol[1] and several diterpenes in the wood.

Further, from point of view on the structural basis skeleton, it is noticed that three types of diterpene, e.g. isodextropimaric acid (A type in Fig. 1), ferruginol and others (B type), and phyllocladene (C type), are found together in the wood extract.



(I) (II) (III)

(A) (B) (C)

FIG. 1. Basic Skeleton of Diterpenes from *Cryptomeria japonica* D. Don.

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[2] E. Mosettig and W.R. Nes, *J. Org. Chem.* **20**, 884 (1955).
[3] J. Simonsen, (Ed.), The Terpenes, Vol. III, 340 (1952).
[4] C.W. Brandt, *Chem. Abst*, **33**, 551 (1939).

* The nomenclature referred in this paper is based upon the same numbering as that of the diterpenoid alkaloids[6].

[5] J.D. Cocker and T.G. Halsall, *J. Chem. Soc.*, **1956**, 4262.
[6] K. Wiesner, J.R. Armstrong, M.F. Bartlett and J.A. Edwards, *J. Am. Chem. Soc.*, **76**, 6068 (1954).
[7] L.H. Briggs and M.D. Sutherland, *J. Org. Chem.*, **13**, 4 (1948).
[8] J.D. Cocker, T.G. Halsall and A. Bowers, *J. Chem. Soc.*, **1956**, 4259.

EXPERIMENTAL

Isolation Procedure.

Phyllocladanol was isolated from the methanol extract of heartwood meal of *Cryptomeria japonica* in the manner described in the previous paper of this series[1]. In a few separate experiments tedious isolation methods were made to isolate the diterpene alcohol from the neutral light petroleum-soluble fraction of the methanol extract without application of the saponification procedure. A pale yellow viscous liquid which was obtained as the residue after steam-distillation of the neutral fraction was absorbed from light petroleum on alumina. Elution with light petroleum yielded a ferruginol fraction, further prolonged elution with ether gave β-sitosterol fraction followed by a fraction containing a small quantity of phyllocladanol. On evaporation of the solvent a solid almost colorless, was obtained. Purification of the product from methanol gave needles, m.p. 182°. Accordingly, a part of the product is at least presented in a chemical free state in wood.

Phyllocladanol.

Colorless needles, m.p. 182~183°, $[\alpha]_D^{17}+15.3°$ (c, 0.99 in MeOH). Found: C, 83.03; H, 11.60. Calcd. for $C_{20}H_{34}O$: C, 82.69; H, 11.80%. No ultraviolet absorption in methanol solution was observed over the range of 220~400 mμ. It was readily soluble in ether and chloroform, sparingly soluble in methanol, ethanol and acetone, soluble in cold sulfuric acid to a colorless solution, and was sublimable under diminished pressure. The Liebermann-Burchard test and the tetranitromethane reaction in chloroform solution were negative, respectively. The acetone solution did not give any color-change with potassium permanganate solution, but the chloroform solution slowly absorbed bromine. There was no uptake of hydrogen when a solution in acetic acid was shaken with a platinum oxide catalyst, indicating the absence of simple olefinic unsaturation. The infrared bands (KBr tablet) were found at 3436 and 1126 (tertiary OH), and 1397, 1383, 1172, 1152 and 920cm^{-1} (*gem*-dimethyl group) (no double bond absorption). On acetylation with acetic anhydride or benzoyl chloride in pyridine, phyllocladanol was recovered unchanged. A mixture of phyllocladanol, anhydrous sodium acetate and acetic anhydride was refluxed for 2 hrs. Working up in the usual manner gave a product as fine needles, m.p. 91~93°, on which analytical figures showed it to be a mixture of anhydro-compound and monoacetyl derivative. Found: C, 85.56; H, 11.37. Calcd. for $C_{22}H_{36}O_2$ (monoacetate): C, 79.46; H, 10.92. Calcd. for $C_{20}H_{32}$ (anhydro-compound): C, 88.16; H, 11.84%.

Chromic Acid Oxidation . . .

A solution of phyllocladanol (100 mg) in 12 ml of the oxidizing solution containing 60 mg of chromium trioxide was kept overnight in an ice-chamber. Excess chromic acid was reduced with methanol and the solution was poured into a large amount of water. The chloroform extract gave a starting material, m.p. 182°, (20 mg).

Dehydration of Phyllocladanol . . .

(A) Phyllocladanol (200 mg) in pyridine (10 ml) was treated with phosphoryl chloride (0.6 ml). After a lapse of 25 hr. at −5° it was added dropwise, with stirring, to ice and water. A white precipitate was formed, filtered and recrystallized several times from a mixture of ether and methanol as leaflets or prisms, m.p. 98°, $[\alpha]_D^{15}+16.6°$ (c, 0.99 in $CHCl_3$). Found: C, 88.03; H, 11.62. Calcd. for $C_{20}H_{32}$: C, 88.16; H, 11.84%. IRλ_{max}^{KBr} 3058, 1653 and 867 cm^{-1} (vinylidene group); 1391, 1374, 1145 and 920 cm^{-1} (*gem*-dimethyl group). These features to the dehydration product agreed with those of phyllocladene[3].

(B) A solution of 50 mg of phyllocladanol in 20 ml of 5% alcoholic sulfuric acid was refluxed for 20 min.. The resulting colorless solution was added to a large volume of water. The product weighed 40 mg and after recrystallization from methanol melted at 108°, showing no depression in melting point on admixture with that obtained in a manner as described below.

(C) A powdered mixture of phyllocladanol (300 mg) and anhydrous potassium hydrogensulfate (1 g) was heated for 1 hr. at 190° in an oil bath. To a brownish reaction mixture was added a large amount of water. Ether extraction gave a solid which was absorbed from ether on alumina. Elution with the same solvent gave isophyllocladene as prisms (crystallized three times from ether-methanol), m.p. 108°, $[\alpha]_D^{13}+22.2°$ (c, 0.87 in $CHCl_3$). Found: C, 88.30; H, 11.69. Calcd. for $C_{20}H_{32}$: C, 88.16; H, 11.84%. Further identification of the product as isophyllocladene was made through preparation of its derivatives mentioned below.

Isomerisation of Dehydration Product (m.p. 98°, II).

Anhydro-compound, m.p. 98°, which was prepared by the above-mentioned phosphoryl chloride method, was treated with 5% alcoholic sulfuric acid on a water bath. Working up in the usual manner gave a product, m.p. 108°, which was identified by melting point and mixed melting point determinations with isophyllocladene.

Identification of Dehydration Product (m.p. 108°, III).

α-Dihydrophyllocladene . . .

Anhydro-compound, m.p. 108°, (50 mg) in acetic acid (5 ml) was hydrogenated at atmospheric pressure in the presence of Adams' catalyst (30 mg). After removal of the catalyst, evaporation under reduced pressure gave a solid. The residual solid was recrystallized several times from ethanol to give a dihydro-compound as leaflets, m.p. 72∼74°, $[\alpha]_D^{9}+26.8°$ (c, 0.38 in $CHCl_3$). Found: C, 87.64; H, 12.32. Calcd. for $C_{20}H_{34}$: C, 87.51; H, 12.49%. These physical constants were in agreement with those of α-dihydrophyllocladene3). The mother liquor gave a second crop, m.p. 55∼57°, which corresponded to β-dihydrophyllocladene, but lack of material did not permit further characterization.

Isophyllocladene Dibromide . . .

To a solution of the anhydro-compound, m.p. 108°, (50 mg) in ether (2 ml) was added dropwise ethereal bromine solution at ice-cooled temperature until the color of the bromine was discharged. The mixture was then evaporated under an ice-cooled condition and a solid isolated, to give dibromide as colorless needles (from ether-methanol), m. p. 149∼151° (decomp.). Found: Br, 35.43. Calcd. for $C_{20}H_{32}Br_2$: Br, 36.97%.

Occurrence of Isophyllocladene . . .

A steam-volatile oil (5.6 g) obtained from methanol extract of the wood meal was subjected to fractional vaccum distillation. The distillation gave fraction, (a) b.p. 100∼160° (bath temp.)/0.4 mm (4.7 g), (b) b. p. 160∼180°/0.4 mm (0.5 g) and (c) residue (0.4 g). The second fraction (b) deposited a crystalline mass after it was kept for a few days at room temperature. The mass filtered and on repeated recrystallizations from benzene-methanol gave a product, white leaflets (200 mg), m.p. 108°, $[\alpha]_D^{15}+23.0°$ (c, 0.91 in $CHCl_3$). Found: C, 88.25; H, 11.73. Calcd. for $C_{20}H_{32}$: C, 88.16; H, 11.84%. A preliminary examination of the infrared spectra of the compound suggested that this was isophyllocladene7). This was confirmed by a mixed melting point determination with the specimen mentioned above.

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Studies on L-Glutamic Acid Fermentation

Part I. Isolation of a L-Glutamic Acid Producing Strain and its Taxonomical Studies

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Screening tests were carried out in order to obtain microorganisms which are able to produce L-glutamic acid from glucose and nitrogen sources.

Five strains having exceedingly high L-glutamic acid productivity were obtained.

Most of these strains were found to belong to the Genus *Brevibacterium*. "*Brevibacterium divaricatum* nov. sp." was the name proposed by the authors for the new group of these strains.

INTRODUCTION

It has been the common commercial processes to manufacture L-glutamic acid either by isolation of hydrolyzed protein such as soybean cake and wheat gluten or by cracking down the pyrolidone-carboxylic acid chiefly resulting from Steffen's molasses. However the yields of L-glutamic acid obtained by these methods largely depend on the L-glutamic acid content in the raw materials used.

On the other hand, chemical synthesis[1~5] has been applied to the preparation of glutamic acid; but the product obtained is always the DL-form which have to be separated from each other by rather difficult and expensive methods.

Generally speaking, the synthesis of L-glutamic acid in various biological systems has been known as either by L-glutamic dehydrogenase or by transaminase; and in both reactions α-ketoglutaric acid is taken as the direct precursor.

In other words, by making use of the above reactions it is quite possible to prepare L-glutamic acid by fermentation.

In case of transamination, the other amino acid is required as the amino donor, that means it could be a valuable industrial process if only cheap amino donors are available in large quantity. In order to make the amination by L-glutamic dehydrogenase proceed in the direction of reduction, there should exist another dehydrogenase system to offer some reduced coenzyme as well as the hydrogen donor.

Moreover, remenbering the fact that α-ketoglutaric acid is a member of Tri-carboxylic acid (TCA) cycle, it follows that it is not impossible to produce L-glutamic acid by fermentation from also other members of the TCA cycle, or glucose and starch which cost cheaper.

In recent years, a new practical method for the preparation of L-glutamic acid has been devised. This method is concerned with the incubation of certain microorganisms in the medium containing glucose, ammonium salt and minerals.

Asai et al.[6] reported the discovery of better productive strains among those which belong to *Micrococcus varians*, and *Bacillus megaterium*.

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1) H. Adkins, *J. Am. Chem. Soc.*, **71**, 3051 (1949).
2) Du Pont, U.S. Patent, 2,437, 600 (1948).
3) Badlisch, German Patent 839, 801 (1952).
4) S. Imoo, *J. Chem. Soc. Japan*, **77**, 1048 (1956).
5) T. Ogawa (to Ajinomoto Co.), Japan Patent 2, 972 (1956).
6) T. Asai, K. Aida and K. Oishi, This Bulletin, **21**, 134(1957).

Kinoshita et al.[7,8] also reported on many microorganisms; especially, one strain named *Micrococcus glutamicus* nov. sp. produced more than 30 per cent L-glutamic acid for glucose supplied, even at a 10 per cent glucose concentration.

This successful result has suggested the possibility of preparing other amino acids by fermentation.

The authors have attempted to screen the new microorganisms which would produce L-glutamic acid directly from glucose resulting in the discovery of *Brevibacterium divaricatum* nov. sp., which gave a higher yield. This paper is concerned with the distribution of L-glutamic acid producing strains in nature and some taxonomical studies of this new strain.

EXPERIMENTAL AND RESULTS

(I) Isolation of L-glutamic acid producing strains

Microorganisms were chiefly isolated from sewage and soils obtained from such places as fields, forests, greens, and reclaimed grounds. A sample of 0.1 g was suspended in 5 ml of sterilized water, and then cultivated with agar medium, composed of the materials mentioned in Table I, on the plate by ordinary way. The colony formed after 48 hours cultivation at 28～30°C was inoculated to the another slant culture (the same composition). It was inoculated into the 5 ml of liquid medium, whose composition is shown in Table II, inside the test tube after having completed growth. Cultivation was performed at 30°C on a reciprocating shaker at a rate of 112 r. p. m.

TABLE I. CULTURE MEDIUM EMPLOYED FOR ISOLATION

Glucose	2.0 %
NH_4Cl	0.4
K_2HPO_4	0.1
$MgSO_4 \cdot 7H_2O$	0.05
Casate	0.02
$CaCO_3$	0.5
Distilled water : Tap water	(1 : 1)
pH	7.0

TABLE II. COMPOSITIONS OF MEDIA FOR THE SCREENING TESTS

Medium Composition	A	B	C
Glucose	5.0 %	5.0 %	5.0 %
NH_4Cl	1.5	—	—
$(NH_4)_2SO_4$	—	2.0	—
Urea	—	—	0.8
K_2HPO_4	0.1	0.1	0.1
$MgSO_4 \cdot 7H_2O$	0.05	0.05	0.05
Peptone	0.2	0.2	0.2
Meat extract	0.2	0.2	0.2
$CaCO_3$	3.0	3.0	—
pH	7.0	7.2	7.2

TABLE III. DISTRIBUTIONS OF L-GLUTAMIC ACID PRODUCING STRAINS

Sources of sample	Number of samples	Number of strains isolated	L-Glutamic acid producing strains				
			(B)	(S)	(Y)	(F)	Total
Field	125	623	42	19	17	23	101
Forest soils	31	148	11	3	1	15	30
The green	83	394	36	13	4	26	79
Compost, dirt	49	195	17	9	12	14	52
Others	6	24	7	—	—	—	7
Total	294	1384	113	44	34	78	269
Cesspool	54	250	10	—	—	8	18
Paddy fields	11	54	4	—	—	2	6
Total	65	304	14	—	—	10	24
Grand Total	359	1688	127	44	34	88	293
% of L-Glutamic acid producing strains			43.3	15.0	11.6	30.1	

B: Bacteria, S: Streptomycetes, Y: Yeasts, F: Fungi

The microorganisms were removed by a centrifugal

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7) S. Kinoshita, K. Tanaka, S. Udaka and S. Akita, Report presented at the symposia on International Enzyme Chemistry (1957).

8) S. Kinoshita, K. Nakayama and S. Akita, This Bulletin, **22**, 176 (1958).

separator after having been cultivated for 72 hours. To find whither or not L-glutamic acid was accumulated, the broth was subjected to test with paper chromatography. A mixture of *n*-butanol, acetic acid, and water (4 : 1 : 1 by vol.) was employed as the developing solvent. A 0.1% ninhydrin butanol solution was employed to color the spot of paper chromatogram. The standard L-glutamic acid solution was always developed on the paper at the same time while the culture broth was tested. From the size and density of the resulted spots, not only the presence of L-glutamic acid was identified but the concentration was also conjectured. For the quantitative analysis of L-glutamic acid, the manometric method using a glutamic decarboxylase preparation obtained from *E. coli* najjar was adopted.

A brief conception of the distribution of L-glutamic acid producing strains in the microbial field is given in Table III. The isolated strains were 1688 in total. Of which 293 strains had been giving spots of L-glutamic acid. The accumulating ability of these strains are shown in the Table IV. The ability was measured by the amount of L-glutamic acid accumulated throughout 72 hours' cultivation.

TABLE IV. ACCUMULATING ABILITY

L-Glutamic acid accumulating ability	Number of strains isolated	%
⧻	5	1.7
⧺	23	7.8
+	265	90.5
Total	293	100.0

Symbols used in the table:
⧻: more than 60 μM/ml.
⧺: between 60 μM/ml and 20 μM/ml.
+: less than 20 μM/ml.

As it is evident from the table, such strains are considerably widely distributed among various genera, and from this fact it may well be considered that the ability to produce and accumulate L-glutamic acid to a certain extent is one of the general and fundamental physiological properties of these microorganisms. However, strains which have the ability to accumulate large amounts of L-glutamic acid were rather rare, and moreover, the amounts produced varied according to the composition of medium and the conditions of fermentation employed.

Although in most cases, L-glutamic acid accumulated was accompanied with L-alanine and L-aspartic acid, in several occasions it was found to accompanied by L-leucine, L-isoleucine, and L-valine.

The screening tests were successful in obtaining five strains of exceedingly high L-glutamic acid productivity. The strain No. 1627, which was among those that possessed especially high L-glutamic acid accumulating ability, has been identified to belong to the Genus *Brevibacterium*. A taxonomical study of this strain will be shown in the following paragraphs:

(II) Taxonomical studies of strain No. 1627

Diagnostic tests were carried out according to the "Manual of methods for pure culture study of bacteria" 6th Ed. 1950, Society of American Bacteriologists, and referred to "Bergey's Manual of Determinative Bacteriology" 7th Ed. 1957, and "Jitken Nōgei-Kagaku" 6th Ed. 1957, University of Tokyo.

Temperature for incubation of bacteria was generally maintained at 37°C in most cases, except in the case of gelatin liquefaction tests. The other tests were carried out in the usual way.

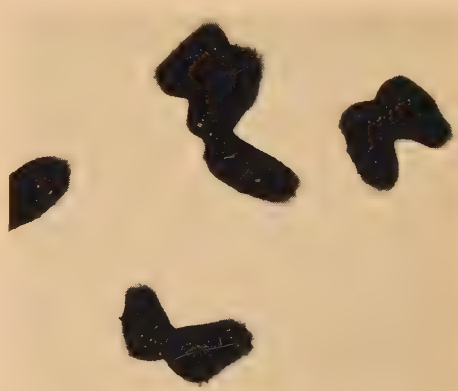

FIG. 1. Normal Form (×8000)

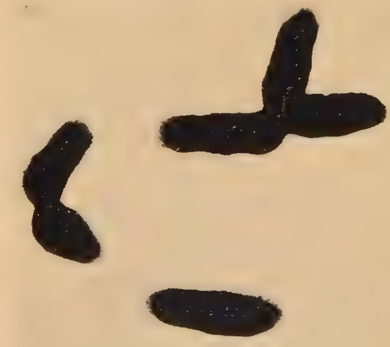

FIG. 2. Elongated Form (×8000)

(1) The properties of strain No. 1627

I) Microscopic observation: Cells are straight rods with rounded ends, 0.8 to 1.0, by 1.5 to 2.0 micron. Usually single, occasionally in pairs or irregular masses. Elongated forms of 2.0 by 6.0 microns are often seen. No capsules or endospores observed. Non-motile, no flagella and gram-positive (Fig. 1 and Fig. 2).

II) Agar colonies: Abundant growth, punctiform, smooth, raised, glistening, yellowish-gray, non-viscid. Edge is entire.

III) Agar slant: Moderate growth, pale yellowish-gray, beaded to filiform, glistening, non-viscid. Medium unchanged.

IV) Gelatin stab: None or scanty liquefaction.

V) Nutrient broth: Slightly turbid, yellowish-brown flocculent sediment, no odor. Amount of sediment is abundant.

VI) Physiological properties:

1. Temperature relations:

Optimum temperature	28～30°C
Maximum temperature	37°C
Minimum temperature	10°C

Thermal death point at 62°C for 10 minutes.

2. Litmus milk:
Slightly acid production. Soft coagulum is formed. No peptonization on the 3rd day. Reduction of litmus begins on the 4th day, ends on the 14th day.
3. Indole is not produced.
4. Nitrates not reduced to nitrites.
5. Ammonia is not produced.
6. Hydrogen sulfide is not produced.
7. Methyl red test, negative.
8. Voges-Proskauer test, negative.
9. Catalase, strongly positive.
10. Urease positive.
11. Reaction to free oxygen: aerobic.
12. pH range: Optimum pH 7.0 to 8.0.
13. Biologic relationships: saprophytic.
14. L-Glutamic acid is accumulated in a large quantity, aerobically in the presence of carbohydrates, ammonium ion and inorganic salts.
15. Acid formation from carbohydrates: As shown in Table V, acid is produced from glucose, fructose, mannose, sucrose, maltose and glycerol. In all cases gas formation is not recognized.
16. Source: Isolated from uncultivated soil.

(2) Decision of family

Our strain is gram-positive, unbranched straight rod, and incapable to form endospore. Therefore, it belongs to Family IX *Brevibacteriaceae* described in Bergey's Manual 7th Ed.

TABLE V. ACID PRODUCTION EROM CARBOHYDRATES BY STRAIN NO. 1627

	Carbohydrates	Acid Production	Gas in peptone water
a)	Monosaccharides		
	Arabinose	–	–
	Rhamnose	–	–
	Xylose	–	–
	Glucose	+	–
	Fructose	+	–
	Galactose	–	–
	Mannose	+	–
b)	Disaccharides		
	Lactose	–	–
	Sucrose	+	–
	Maltose	+	–
	Trehalose	–	–
	Melibiose	–	–
	Cellobiose	–	–
c)	Polysaccharides		
	Raffinose	–	–
	Starch	–	–
	Inulin	–	–
	Dextrin	–	–
	Glycogen	–	–
d)	Alcohols		
	Glycerol	+	–
	Arabitol	–	–
	Adonitol	–	–
	Mannitol	–	–
	Sorbitol	–	–
	Dulcitol	–	–
e)	Glucosides		
	Salicin	–	–

+ : Acid or gas produced.
– : Acid or gas not produced.

Our strain has a rather short form, and is non-pathogenic. If our strain is regarded as wedge or club forms, and pathogenic on animals or plants, then there is a possibility that it belongs to Family XII *Corynebacteriaceae*. However, members of this family are usually pathogenic and all species described in this family are not in accord with our strain.

If our strain is to be regarded as ellipsoidal form, *Micrococcus candidus* and *Staphylococcus epidermidis* would be comparatively related with our strain, but the former is different from our strain in its acid production from carbohydrates, and the latter is different in action on nitrates and its amino acids requirement.

(3) Decision of genus

In *Brevibacteriaceae*, there are two genera, i.e., *Brevibacterium* and *Kurthia*. *Kurthia* contains only three species, i. e., *Kurthia zopfii*, *Kurthia variabilis* and *Kurthia bessonii*. According to the descriptions in Bergey's Manual, *Kurthia* is motile with peritrichous flagella, facultatively anaerobic and has no action on carbohydrates.

Our strain is non-motile, no flagella and capable to attack carbohydrates. Consequently, our strain cannot be included in the above three species. In case of *Brevibacterium*, the following descriptions can be found in Bergey's Manual: "Typically short, unbranching rods. Generally non-motile. May or may not reduce nitrates. Acid usually produced from simple carbohydrates (glucose broth usually becomes acid; lactose not fermented). Aerobic and facultatively anaerobic."

Our strain conforms to these descriptions. Thus, our strain was confirmed to belong to the genus *Brevibacterium*.

(4) Decision of species

As our strain is non-motile, so *Brevibacterium incertum*, *Brevibacterium imperiale*, *Brevibacterium lipolyticum*, *Brevibacterium acetylicum*, *Brevibacterium sulfureum*, and *Brevibacterium helvolum* are differentiated from our strain in their motile character. *Brevibacterium linens*, *Brevibacterium erythrogenes*, *Brevibacterium fulvum*, *Brevibacterium insectiphilium*, *Brevibacterium brunneum*, *Brevibacterium vitarumen*, *Brevibacterium maris*, and *Brevibacterium fuscum* are different from our strain in their red, rose, or orange pigment formation on agar.

TABLE VI-1. COMPARATIVE STUDIES ON PHYSIOLOGICAL CHARACTERISTICS OF STRAIN No. 1627 AND OTHER BREVIBACTERIUM SPECIES

Strain \ Tests	Motility	Relation to free oxygen (+ : aerobic)	Nitrate reduction	Litmus milk	Gelatin liquefaction	Indole formation	Hydrogen sulfide production
Brevibacterium linens	-	+	+	Al	+		+
Brevibacterium erythrogenes	−	+	+	A	s+	±	+
Brevibacterium fulvum	−	+	−	A	s+	+	
Brevibacterium insectiphilium	−	+	−	Al	+	−	−
Brevibacterium brunneum	−		−	N	+	−	
Brevibacterium vitarumen	−		+	A	−	−	
Brevibacterium maris	−	+	+	Al→A	−	−	−
Brevibacterium fuscum	−	+	+	A→Al	s+	−	
Brevibacterium minutiferula	−	+	−	N→A	−	−	−
Brevibacterium sociovivum	−	+	−	N	+	-	−
Brevibacterium immotum	−	+	−	N	+	−	−
Brevibacterium marinopiscosum	−	+	−	N	+	−	−
Brevibacterium tegumenticola	−		−	N	−	−	−
Brevibacterium stationis	−	+	+	Al	s+	−	−
Brevibacterium quale	−		+	N	+	−	−
Brevibacterium ammoniagenes		+	+	Al	−	−	
Brevibacterium healii	−			A	+	−	
Brevibacterium incertum	+		-	N	−		
Brevibacterium imperiale	+	+	−	N→A	−	-	
Brevibacterium lipolyticum	+	+	±	Al	+	±	−
Brevibacterium acetylicum	+	+	−	A	+	−	
Brevibacterium sulfureum	+	+	−	Al	+	−	
Brevibacterium helvolum	−	+	−	A→Al	s+	−	+
Strain No. 1627	−	+	−	A	±	−	−

N: no change, A: acid, Al: alkaline, s: slow

Our strain is also different from *Brevibacterium stationis*, *Brevibacterium quale*, and *Brevibacterium ammoniagenes* in the respect that these species can reduce nitrate to nitrite. *Brevibacterium minutiferula*, *Brevibacterium sociovivum*, *Brevibacterium immotum*, and *Brevibacterium marinopiscosum* are different from our strain in their sea water requirement for initial growth. Therefore, only *Brevibacterium tegumenticola* and *Brevibacterium healii* can be considered as species related with our strain. However, these species are found to be different from our strain in the following characters:

	Brevibacterium tegumenticola[9]	*Brevibacterium healii*	Strain No. 1627
Agar colonies	Tiny, white, convex	Large, white, rhizoid	Punctiform, yellowish-gray, raised
Gelatin stab	Generally no liquefaction	Stratiform liquefaction	None or scanty liquefaction
Broth	Slight turbidity	No turbidity	Slight turbidity
Litmus milk	No change	Slightly acid, peptonized	Slightly acid, not peptonized
Acid production from carbohydrates	Glucose, maltose, sucrose	Glucose, fructose, maltose, sucrose, salicin, starch	Glucose, fructose, mannose, sucrose, maltose, glycerol
Source	Integument of the bed-bug	Dairy products	Soil

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9) Steinhaus, *Jour. Bact.*, **42**. 775 (1941).

TABLE VI-2.

Strain \ Carbohydrates	Arabinose	Rhamnose	Xylose	Glucose	Fructose	Galactose	Mannose	Lactose	Sucrose	Maltose	Trehalose	Rhaffinose	Starch	Inulin	Dextrin	Glycerol	Adonitol	Mannitol	Sorbitol	Dulcitol	Salicin
Brevibacterium linens	–	–	–		–			–	–	–		–		–				–	–		–
Brevibacterium insectiphilium	–	–	–	–	–	–	–	–	–	–	–	–		–	–	–	–	–	–	–	–
Brevibacterium brunneum				–				–	–												
Brevibacterium vitarumen			–	+				–	+	+								–	–	–	
Brevibacterium maris				+				–	–												
Brevibacterium minutiferula				+				–	+	–											
Brevibacterium sociovivum				+				–	–	+						–		+			–
Brevibacterium immotum			+	+				–	–	+						–		+			–
Brevibacterium marinopiscosum			–	+				–	–	–						–		+			–
Brevibacterium tegumenticola				+				–	+	+											
Brevibacterium stationis			–	+				–	–	–						–		–			–
Brevibacterium quale				+				–	+	+											
Brevibacterium ammoniagenes	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Brevibacterium healii				+	+			–	+	+		–	+	–				–			+
Brevibacterium incertum		–		+	+	–	+	–		+								–	–	–	
Brevibacterium imperiale	+	–	+	+	+	+	+	±	+	+	+	+		–	±	–	–	+	+	–	+
Brevibacterium lipolyticum			+	+				–	+			+				+		+			
Brevibacterium acetylicum				+																	
Brevibacterium sulfureum				–																	
Brevibacterium helvolum	+			+	+	+			+							+		+			
Strain No. 1627	–	–	–	+	+	–	+	–	+	+	–	–	–	–	–	+	–	–	–	–	–

The comparative studies between named species in Bergey's Manual, 7th Ed. and our strain No. 1627 are presented in Table VI-1 and Table VI-2.

From the above experimental facts, there are no species which conform to our strain appearing in Bergey's Manual 6th Ed. and 7th Ed., so our strain No. 1627 seems to be of a new species.

Therefore, we propose assign the name, *Brevibacterium divaricatum* nov. sp. to our strain, in relation to its marked arrangement of cells which exist in the stretching form.

Acknowledgement The authors wish to express their sincere thanks to Prof. T. Asai for his kind advice throughout this work.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 1, p. 75~83, 1960]

Effect of Gamma-ray upon Food Microorganisms

Part IV. Studies Concerning Influences of Amino-acids and Proteinous Materials upon Survival of *E. coli* by Gamma-ray Irradiation

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Effects of the addition of amino-acids and proteinous materials to a medium on the survival of *E. coli* which was irradiated with Cobalt-60 gamma-ray were investigated. This report deals with a comparison of the survival-curves obtained with media containing each of fifteen kinds of amino-acid and three different proteinous material.

INTRODUCTION

For the last ten years, much progress has been made in the application of ionizing radiations to the preservation of foodstuffs, i. e., the so-called "Cold Sterilization." Nowadays, the possibility of applying radio-active rays for long term preservation of food-stuffs without refrigeration has been recognized by many workers[1~5]. However, there are still many unknown questions concerning the sterilizing effect of radio-active ray upon food microorganisms under different conditions. In the case of irradiation of food-stuffs of which a majority contain large amounts of protein, influences of the addition of not only proteins but also free amino-acids upon the survival of microorganisms irradiated with radio-active ray should be taken into consideration. Though there are many works concerning the effects of the addition of chemical substances, very little is known about the influences of amino-acids and proteins upon microorganisms in sterilized food-stuffs[6,7].

The author has carried out studies concerning a simplified method to investigate the sterilizing effect of Cobalt-60 gamma-ray upon *E. coli*[8~10], and this report deals with studies on the influences of the addition of fifteen amino-acids and also three proteinous materials upon the survival-ratios of the same strain of *E. coli.*

EXPERIMENTAL

The strain used in this experiment was *Escherichia coli* strain YC 1, one of the stock cultures preserved at the School of Medicine, Keio University, the same strain as that in three previous resports. The medium used in Experiment 1 was a minimum medium reported by Davis, the components of which were described in detail in Report III. This medium was employed, because some free amino-acids should be contained in nutrient agar medium used in previous experiments. The media

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1) W. Huber, *Electronic Preservation of Foods, Elet.*, **21**, 74 (1948).

2) L.E. Brownell and et al, Utilization of the Gross Fission Products, Progress Report 1-2, Univ. of Michigan (1951).

3) B.E. Proctor and et al., An Investigation of Factors relating to the Processing of Fruits and Vegetables treated by Supervoltage Cathode Rays, Progress Report, M. I. T. (1953).

4) W.M. Urbain, Facts about Cold Sterilization, *Food Tech.*, **25**, (3), (1953).

5) G.B. Prott and O.F. Eklund, Radiation Sterilization of Foods, Quick Frozen Foods, (51) May (1954).

6) W.D. Claus, Enhanced lethal effect of X-rays on *E. coli* in the presence of inorganic salts, *J. Exp. Med.*, **57**, 335 (1933).

7) G.E. Stapleton, D. Billen and A. Hollander, The role of enzymatic oxygen removal in chemical protection against X-ray inactivation of bacteria, *J. Bacteriol.*, **63**, 805 (1952).

8) W. Watanabe, Effect of gamma-ray upon food microorganisms-I, This Bulletin, **22**, (2) 68-77 (1958).

9) W. Watanabe, Effect of gamma-ray upon food microorganisms II, This Bulletin, **22**, (4) 255-261 (1958).

10) W. Watanabe, Effect of gamma-ray upon food microorganisms-III, This Bulletin, **23**, (2) 73-77 (1959).

used in Experiment 2 are a nutrient medium and a minimum medium. The radiation source applied was cobalt-60 of 40 Curies placed at the School of Medicine, Keio University.

The amino-acids were chemical reagents for paper-chromatography, prepared by Ajinomoto K. K. Three amino-acids could not be used because of their non-solubility in distilled water; these were *l*-cystine, *dl*-tryptophan and *l*-tyrosine. The amino-acids used here were *dl*-alanine, *l*-arginine, *l*-aspartic acid, *l*-glutamic acid, glycine, *l*-histidine, *l*-hydroxy proline, *l*-leucine, *l*-lysine, *l*-methionine, *l*-phenyl-alanine, *l*-proline, *dl*-serine, *dl*-threonine and *l*-valine. The concentrations of the amino-acids were 0.05% and 0.10%.

The proteinous meterials were horse-serum, yeast extract and lactalbumin. The concentrations were 1, 5 and 10% in the case of horse-serum, 0.01, 0.02 and 0.04% in case of yeast extract (Difco Co.), 0.1, 0.3 and 0.6% in case of lactalbumin (Nutrient Biochemicals Corp., U.S.A.), and 0.5, 1.0 and 2.0% in case of heated horse-serum.

The samples were prepared as follows. Bacteria were centrifuged and washed two or three times with saline and suspended in a phosphate buffer solution at the concentration of 10^3 cells/ml, and the suspension was diluted to 1:10 with melted agar medium held at 40°∼45°C. One ml of the mixture was pipetted into a small petri-dish and solidified at room temperature. These samples therefore contained bacteria at the concentration of 10^2cells/ml in the agar medium.

TABLE I. BACTERIAL CONCENTRATIONS USED IN THIS EXPERIMENT

Amino acid	0.05%	0.10%
Control	542 /ml	542 /ml
Arginine	555	695
Phenyl alanine	467	481
Lysine	558	469
Valine	446	439
Aspartic acid	670	560
Histidine	741	755
Threonine	442	445
Methionine	577	404
Glutamic acid	493	523
Glycine	410	478
Alanine	505	431
Hydroxy proline	450	563
Leucine	465	485
Serine	429	414
Proline	558	611

The samples were irradiated at room temperature lying between 20° to 25°C, for two hours. The non-irradiated control samples were treated in the same way except in the irradiation procedure. After irradiation all samples, irradiated and non-irradiated, were immediately placed into an incubator and incubated at 37°C for 48∼96 hours. The colonies developed were scored with a binocular microscope.

The pH of the minimum medium was 5.6, and as a result of the addition of amino-acids to the medium, the pH value of the medium which contained one kind of amino-acid and a low concentration of *E. coli* became somewhat lower. Table I shows respective bacterial concentrations of each medium in the experiment of amino-acid addition, and each pH value is also shown in Table II. The lowest pH value, 4.2, was observed in a medium containing aspartic acid at the concentration of 0.10%. The results are described in detail in each of the following sections.

TABLE II. pH VALUE OF USED MEDIUM CONTAINED ONE OF DIFFERENT KINDS OF AMINO-ACID AT THE CONCENTRATIONS OF 0.05 AND 0.10%

Amino acid	Nature	0.05%	0.10%
Control	——	5.6	5.6
DL-Alanine	synthetic	5.2	5.0
L-Arginine monohydro chloride	isolated	5.3	5.1
L-Aspartic acid	isolated	4.7	4.2
L-Glutamic acid	isolated	4.7	4.3
Glycine	synthetic	5.0	4.7
L-Histidine monohydrochloride monohydrate	isolated	5.1	5.0
L-Hydroxy proline	isolated	5.0	4.8
L-Leucine	isolated	5.4	5.3
L-Lysine monohydrochloride	isolated	5.0	4.8
L-Methionine	isolated	5.5	5.4
L-Phenyl alanine	isolated	4.9	4.7
L-Proline	isolated	5.0	4.8
DL-Serine	synthetic	5.0	4.7
DL-Threonine	synthetic	5.5	5.4
L-Valine	synthetic	5.2	5.0

RESULTS AND DISCUSSION

(1) **Experiment 1:** In general, more or less protective influences of amino-acids were observed on the survival of the strain irradiated with cobalt-60 gamma-ray except in only one amino-acid, *l*-arginine. However, tendencies of their

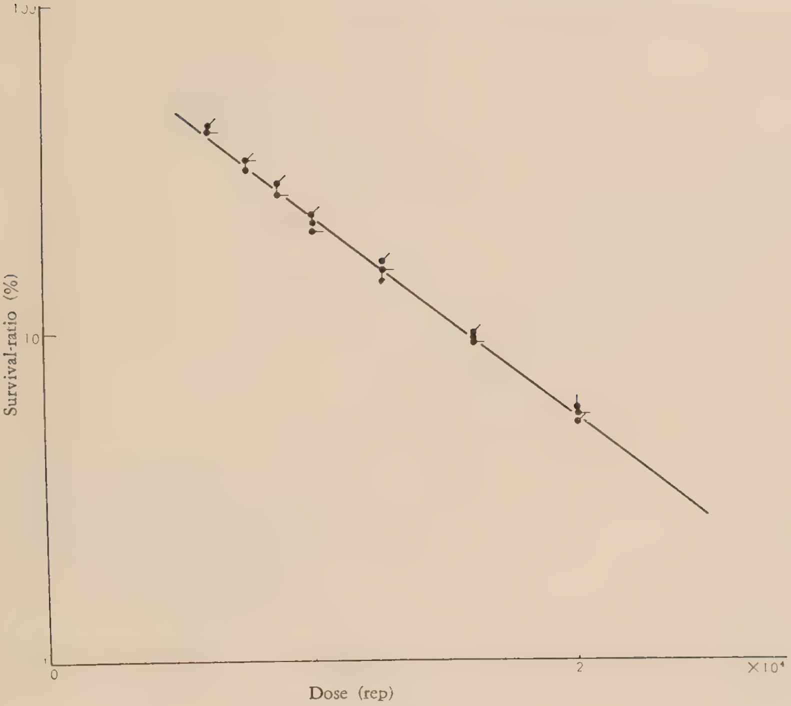

FIG. 1. Influence of Addition of Arginine upon Survivals of *E. coli* by Gamma-ray Irradiation

●| 0 % 615 cells/ml
●/ 0.05 555
●– 0.10 695

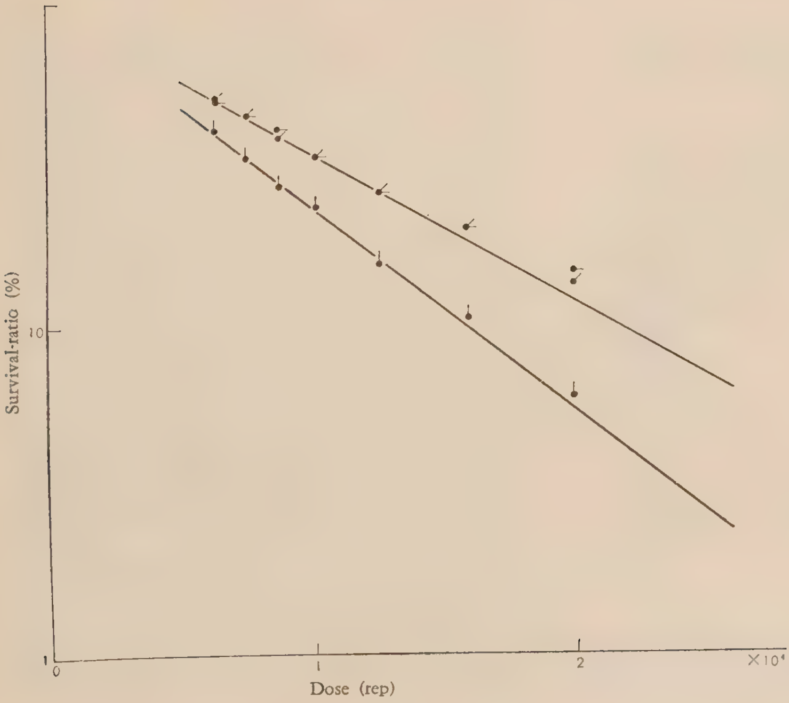

FIG. 2. Influence of Addition of Threonine upon Survivals of *E. coli* by Gamma-ray Irradiation

●| 0 % 476 cells/ml
●/ 0.05 442
●– 0.10 445

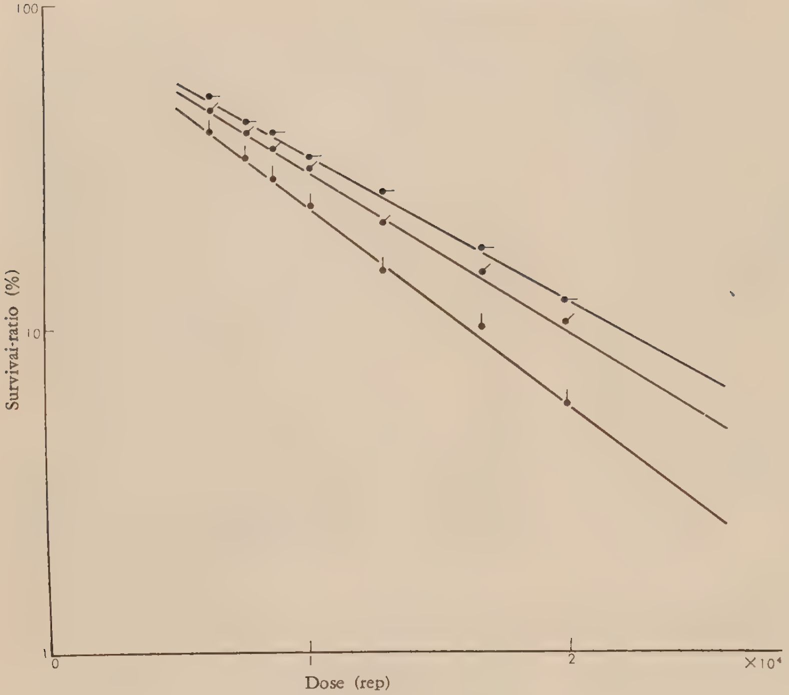

FIG. 3. Influence of Addition of Valine upon Survivals of *E. coli* by Gamma-ray Irradiation

●|	0 %	421 cells/ml
●✓	0.05	446
●–	0.10	439

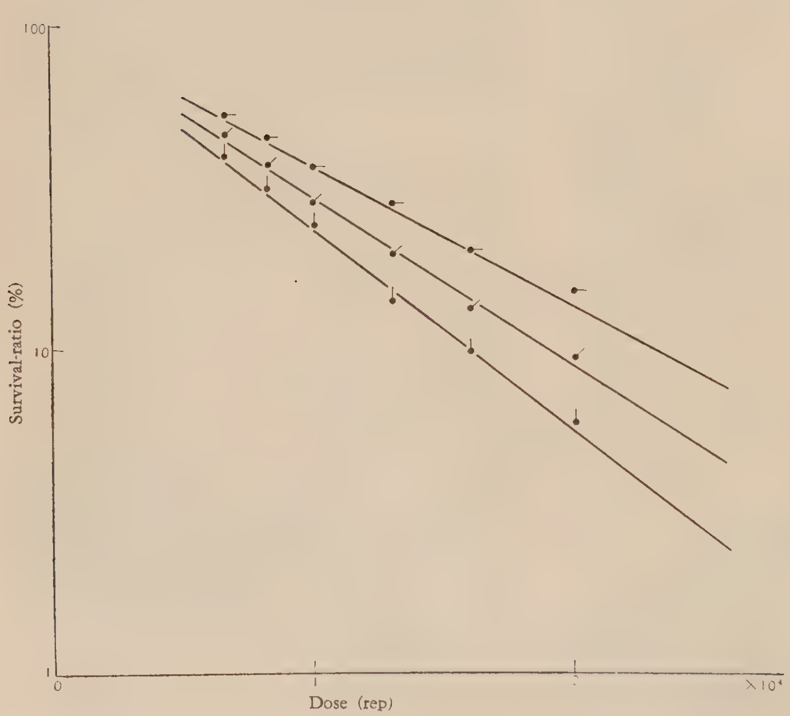

FIG. 4. Influence of Addition of Glutamic Acid upon Servivals of *E. coli* by Gamma-ray Irradiation

●|	0 %	481 cells/ml
●✓	0.05	493
●–	0.10	523

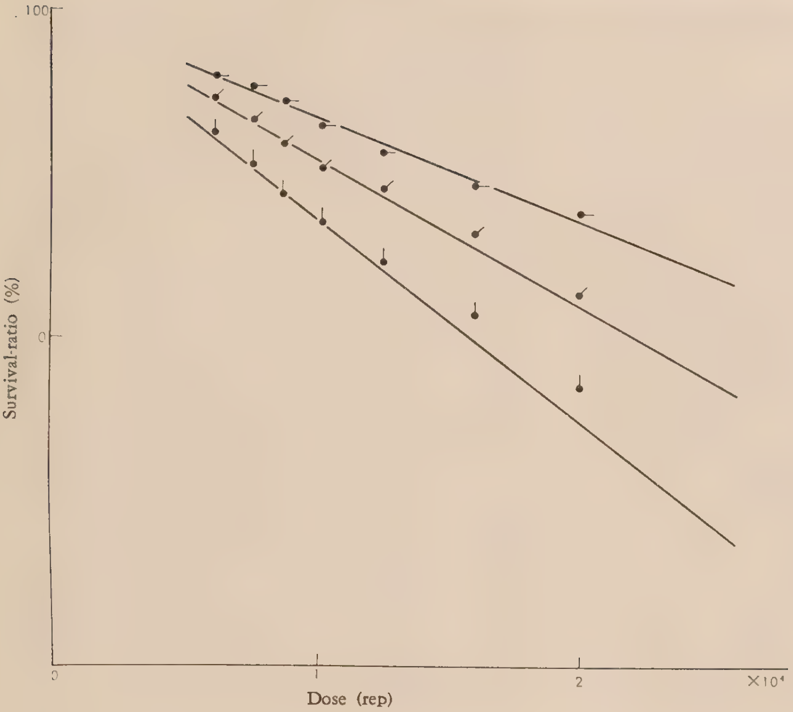

FIG. 5. Influence of Addition of Proline upon Survivals of *E. coli* by Gamma-ray Irradition

● 0	%	592 cells/ml
✓ 0.05		558
●- 0.10		611

protective effect were different from each other. Also, it seemed that there was no correlation between the protective effect and pH value of the medium. Therefore, the amino-acids could be divided into four groups according to their protective effects. The first group had only one amino-acid, arginine, which had almost no influence, as diagrammed in Fig. 1. The second group included phenylalanine, threonine and lysine, whose protective effects were not influenced by both of their different concentrations. The results obtained with threonine are diagrammed in Figure 2. The third group included valine, leucine and histidine, whose protective effects were remarkable. As representative of this group, the results obtained from valine are demonstrated in Figure 3. As it can be seen from the figure, the survival-curve with a 0.10 % was slightly higher than that obtained with 0.05 %. The last group included serine, alanine, glutamic acid, aspartic acid, glycine, methionine, hydroxy proline and proline. In case of these amino-acids, higher concentrations of the amino-acid resulted in higher survival of the bacteria.

TABLE III. COMPARISON OF EACH SLOPE OF SURVIVAL-CURVE OF *E. coli* AS A SIN-VALUE OBTAINED FROM MEDIUM WHICH CONTAINED AMINO ACID

Amino acid	0.05 %	0.10 %
Control	.7834	
Arginine	.7934	.7934
Phenyl alanine	.8090	.8090
Lysine	.8192	.8192
Valine	.8434	.8660
Aspartic acid	.8387	.8704
Histidine	.8526	.8788
Threonine	.8704	.8704
Methionine	.8434	.8870
Glutamic acid	.8387	.8829
Glycine	.8339	.8870
Alanine	.8526	.8910
Hydroxyprolin	.8290	.8829
Leucine	.8704	.9063
Serine	.8788	.9171
Proline	.8746	.9272

The results obtained from glutamic acid are diagrammed in Figure 4 and that from proline in Figure 5.

When all the fifteen survival-curves were diagrammed in the same figure in order to compare the protective effect of each aminoacid, they were so complicated that in both cases of 0.05 and 0.10 % it became difficult to distinguish each other. In Report I, eleven survival-curves were compared by means of calculating each 90 % lethal dose. However, in the present studies, a slope of the survival-curve which is actually a straight line shows the extent of the protective effect. Therefore, each angle between the survival-curve and the vertical line was chosen as the indicator. *Sin*-values obtained from the survival-curves are given in Table III. From this table, the protective effect of each amino-acid upon the survivals of the strain are comparable with various amino-acids.

(2) **Experiment 2:** The protective effects of proteinous materials, both non-heated and heated, were investigated. Horse-serum was chosen as the former, and yeast extract, lactalbumin and heated horse-serum as the latter.

At first, the influence of the addition of horse-serum to the nutrient agar medium was tested. The concentrations of horse-serum were 1, 5 and 10 %. The results are diagrammed in Figure 6. As it can be seen, a weak protective effect is observed. Further, the influence of yeast extract to the same agar medium at concentrations of 0.01, 0.02 and 0.04 % was tested. The results are diagrammed in Figure 7, again showing a weak protective influence. In both cases the addition of proteinous materials to the nutrient agar medium almost no influence on bacterial survival. Such a phenomenon as shown in both cases would be caused by protective substances originally contained in nutrient agar medium at high concentrations. Therefore, it woult be suggested that no additive effect upon survivals of the strain can be expected when a protective substance is added to a medium already containing in high concentrations of the same kind of protective material.

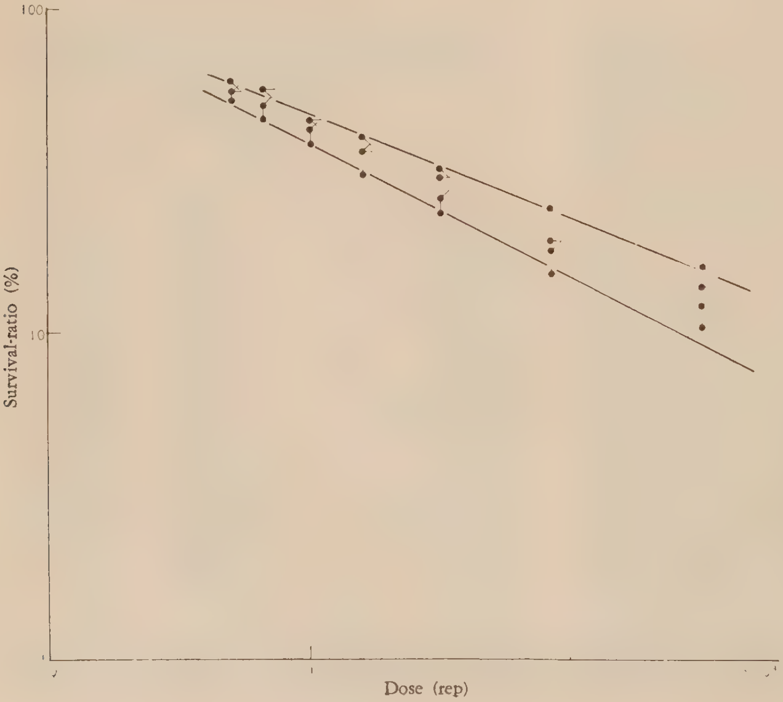

FIG. 6. Influence of Addition of Horse-serum upon Survivals of *E. coli* Contained in a Broth Agar Medium by Gamma-ray Irradiation

●	0 %	597 cells/ml
●	1	577
●-	5	675
●	10	667

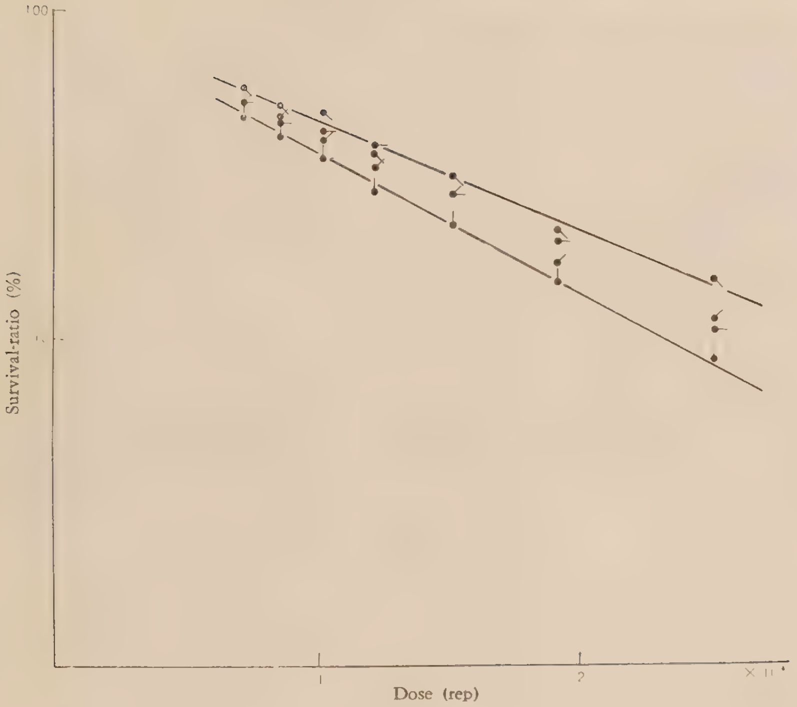

FIG. 7. Influence of Addition of Yeast Extract upon Survivals of *E. coli* Contained in a Broth Agar Medium by Gamma-ray Irradiation

●	0 %	575 cells/ml
●/	0.01	484
●–	0.02	720
●\	0.04	817

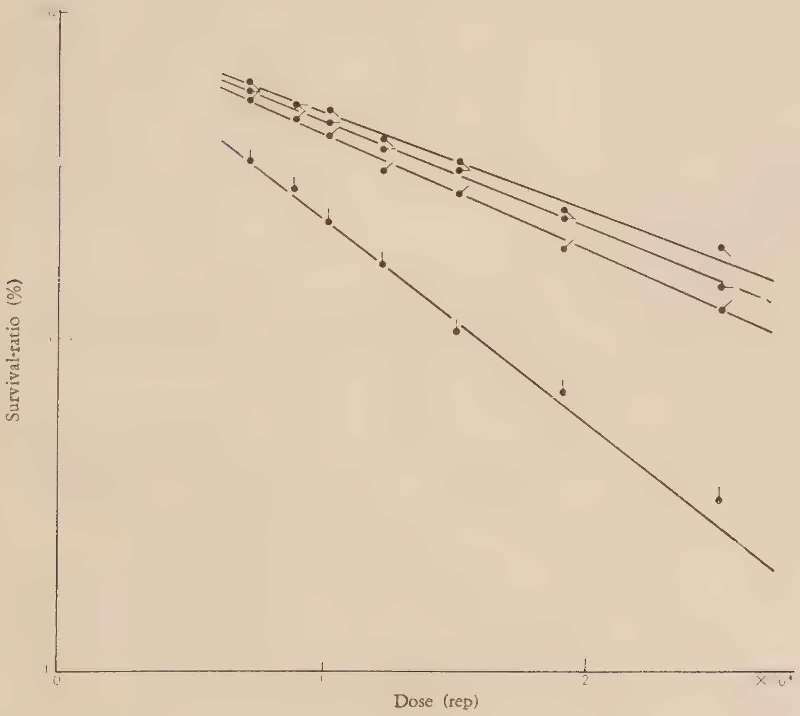

FIG. 8. Influence of Addition of Horse-serum upon Survivals of *E. coli* Contained in a Synthetic Medium by Gamma-ray Irradiation

●	0%	443 cells/ml
●/	1	678
●–	5	545
●\	10	582

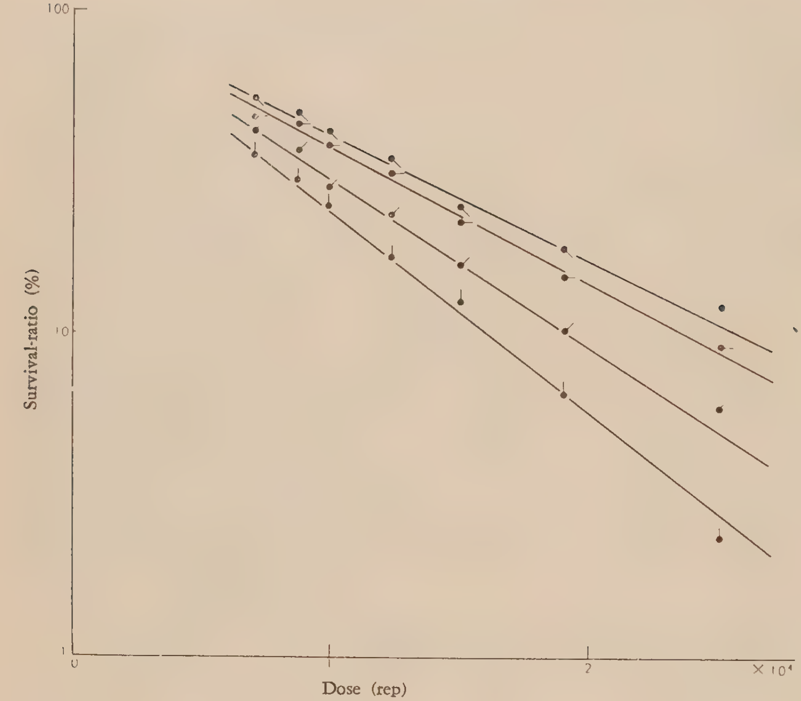

FIG. 9. Influence of Addition of Lactalbumin upon Survivals of *E. coli* Contained in a Synthetic Medium by Gamma-ray Irradiation.

↓	0 %	545 cells/ml
✓	0.1	716
●–	0.3	771
●↖	0.6	821

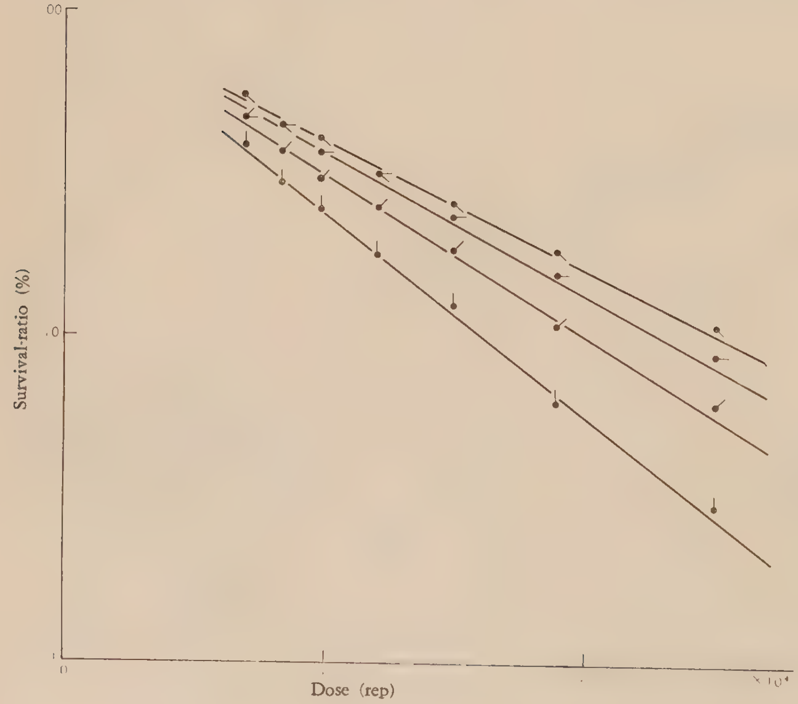

FIG. 10. Influence of Addition of Heated Horse-serum upon Survivals of *E. coli* Contained in a Synthtic Medium by Gamma-ray Irradiation.

↓	0 %	405 cells/ml
✓	0.5	418
●–	1.0	453
●↖	2.0	517

Secondly, the influence of the addition of horse-serum to a minimum medium containing no organic matters was tested. The concentrations of horse-serum used were the same as in the case of nutrient agar medium. The results are diagrammed in Figure 8. As can be seen from this figure, a remarkable protective effect was observed in each case. Further, it was known that the high concentrated horse-serum in higher survival of the bacteria.

Subsequently, the influence of the addition of lactalbumin to a minimum agar medium was tested. The concentrations employed were 0.1, 0.3 and 0.6%. The results are diagrammed in Figure 9. In this case, a protective effect was observed.

Finally, the influence of the addition of heated horse-serum to a minimum medium at concentrations of 0.5, 1.0 and 2.0% was tested and compared with that of non-heated horse-serum. The results are diagrammed in Figure 10. The survival-curve with 1.0% was chosen in order to compare the influences of heated and non-heated horse-serum, each other. One per cent survival-curve in the case of non-heated horse-serum was higher than that of heated serum. Therefore, it would be suggested that non-heated proteinous material would have a more protective effect on *E. coli* irradiated with gamma-ray than heat processed protein.

SUMMARY

(1) The addition of amino-acids to a medium resulted in the protection of *E. coli* irradiated with cobalt-60 gamma-ray.

(2) The tendencies of the protective effects differed from each other. From this viewpoint, they could further be divided into four groups.

(3) In order to compare the protective effect of each amino-acid, the *Sin*-value obtained from each survival-curve was chosen as the indicator.

(4) Proline was found to be most protective, and phenylalanine the least, whereas arginine was not protective.

(5) The addition of proteinous materials also resulted in the protection of *E. coli* irradiated with cobalt-60 gamma-ray.

(6) The addition of proteinous materials to nutrient agar medium resulted in only weak protection. However, their addition to a minimum medium was very protective.

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Effect of Gamma-ray upon Food Microorganisms

Part V. Studies on Survival of *E. coli* irradiated with Gamma-ray

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Received May 11, 1959

The author has carried out a series of studies on the effects of various environmental conditions on the survival of *E. coli* irradiated with gamma-ray. This report deals with the minimum gamma-ray dose-rate necessary for regular survival of the strain under a weak gamma-ray field in three kinds of media, influences of the vacuum procedure, and also, the effects of some other conditions.

INTRODUCTION

In the author's preceeding four reports[1~4], a simplified method for the sterilization of *E. coli* by gamma-ray irradiation has been presented and also, the influences of various conditions prior to, during, and after irradiation on the survival of the strain irradiated with gamma-ray have been reported. However, in regard of each experiment many questions still remain unsolved. In the present investigations, the author has carried out several supplementary studies concerning the effects previously reported, of which the results are presented here.

EXPERIMENTAL

The strain used in this experiment was *Escherichia coli* strain *YC 1*, one of the stock cultures of the School of Medicine, Keio University. The radiation source used Cobalt-60, of 40 Curies, preserved at the School of Medicine, Keio University. The duration of irradiation was 2 hours in most experiments, being occasionally adjusted to the range of from 14 minutes to 9 hours.

The samples were prepared as follows. Bacteria were centrifuged and washed two or three times with saline, suspended in saline at the concentration of 10^3 cells/ml, and the suspension was diluted (1 : 10) with melted agar medium held at 40°～45°C. One ml of the mixture was pipetted into a small petri-dish and solidified at room temperature. Therefore, these samples contained bacteria at the concentration of 10^2 cells/samples ml in the agar medium.

All samples were irradiated at room temperature within the range of 19° to 21°C. The non-irradiated control samples were treated in the same way except in the irradiation procedure. After irradiation, all samples both irradiated and non-irradiated, were immediately placed into an incubator and incubated for 48 hours at 37°C. When a pure agar medium was used for plating, both of the irradiated and non-irradiated control samples were covered with a nutrient agar medium prior to incubation. The colonies developed were scored with a binocular microscope.

The media used were nutrient agar, the minimum medium reported in Report III, and in Experiment 1 a pure agar medium, in Experiment 2 both nutrient agar and a pure agar medium, in Experiments 3, 4 and 5 only a nutrient agar medium, and in Experiment 6 a minimum agar medium. The procedures and results are described in detail in each section below.

RESULTS AND DISCUSSION

(1) **Experiment 1:** In Report III, the author has reported on the relation between the survival-ratio of *E. coli* and dose-rate of cobalt-60 gamma-ray, and has mentioned that the survival-ratio of the strain in nutrient agar medium

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[1] W. Watanabe, Effect of Gamma-ray upon Food Micoorganisms (I), This Bulletin, **22**, 68-77 (1958).

[2] W. Watanabe, 〃 (II), This Bulletin, **22**, 255-261 (1958).

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[4] W. Watanabe, 〃 (IV), This Bulletin, **24**, 75-83 (1960).

is directly proportional to the total irradiated dose in case of irradiation at a dose-rate exceeding 4,000 rep/hr.

In the present experiment, three kinds of media, a minimum medium (synthetic medium), a pure agar medium (non-nutrient) and a nutrient agar medium containing 10 % horse-serum were employed.

At first, twenty petri-dishes containing small numbers of bacteria and a minimum medium were irradiated for varied durations in which almost equivalent total doses of irradiation were administrated to the samples, i. e., 5,000 rep. The relation between the gamma-ray dose-rate and the duration of irradiation is presented in Table I, and the results of the table are also diagrammed in Figure 1. As shown in the figure, the survival-ratios resulted in almost an equivalent value of 48 %, in case of gamma-ray irradiation upon *E. coli* which was carried out in a minimum medium at a dose-rate of more than 2,000 rep/hr. However, in case of irradiation at a dose-rate lower than 2,000 rep/hr, the lower dose-rate of gamma-ray resulted in survival of more bacteria.

TABLE I. DOSE-RATES AND DURATIONS OF IRRAD. OF SAMPLES AT THE DOSE OF 5,000 rep IN CASE OF USING A SYNTHETIC MEDIUM.

Sample No.	Distance from Co^{60}	Dose-rate	Irrad. time	Survival	S-ratio
—	—	0 rep/hr	0 hr	536	100 %
1	5.5 cm	12758	0 h–24 m	263	49.0
2	6.3	9723	0–31	259	48.3
3	7.1	7656	0–40	255	47.6
4	7.9	6210	0–48	264	49.2
5	8.7	5098	0–59	251	46.8
6	9.5	4277	1–10	260	48.5
7	10.3	3638	1–22	251	46.8
8	11.2	3077	1–38	252	47.0
9	12.5	2470	2–02	261	48.7
10	13.7	2057	2–26	249	46.5
11	15.0	1715	2–55	269	50.2
12	16.2	1471	3–24	281	52.5
13	17.5	1259	3–58	285	53.2
14	18.7	1104	4–32	308	57.4
15	20.0	965	5–11	328	61.2
16	21.2	859	5–49	357	66.6
17	22.5	748	6–41	396	73.9
18	23.7	681	7–20	422	78.4
19	25.0	618	8–06	481	89.8
20	26.2	558	8–58	517	96.4

Following this procedure samples containing pure agar were irradiated for varied durations in which the total irradiation doses upon the samples showed almost the same values, i. e., 3,000 rep and 5,000 rep. The durations of irradiation were adjusted in the range of 14 minutes to 5.5 hours in case of 3,000 rep irradiation, and in the range of 24 minutes to 9 hours in case of 5,000 rep irradiation. The results are diagrammed in Figure 2. As shown in the figure, in case of pure agar medium, irradiation at a dose-rate exceeding 1,700 rep/hr resulted in almost the same survival-ratios of the strain assuming the

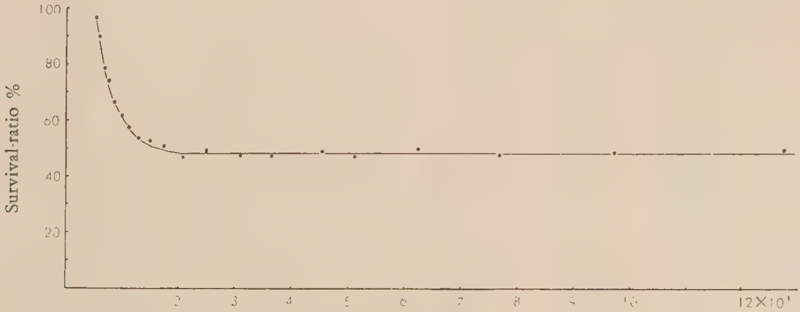

FIG. 1. Relation between Survival-ratio of *E. coli* in Minimum Medium and Dose-rate of Gamma-ray at the Dose of 5,000 rep.

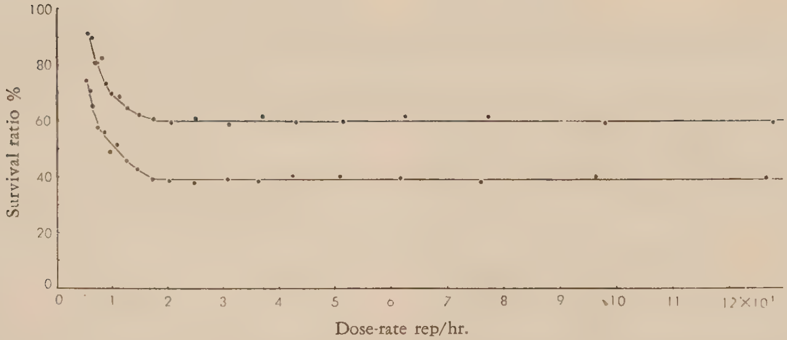

FIG. 2. Relation between Suruival-ratio of *E. coli* in Pure Agar Medium and Dose-rate of Gamma-ray at the Doses of 3,000 and 5,000 rep.

value of 60% in case of 3,000 rep, and 39% in case of 5,000 rep irradiation.

Finally, ten petri-dishes containing a nutrient agar medium enriched with 10% horse-serum were irradiated for varied durations in which the total doses of irradiation upon samples were almost equivalent, i. e., 15,000 rep. The durations of irradiation were adjusted in the range of 1.2 to 7.4 hours. The results are diagrammed in Figure 3. As shown in the figure, in case of a nutrient agar medium enriched with 10% horse-serum at a dose-rate of more than 4,000 rep/hr as only in case of nutrient agar medium in Report III, the survival-ratios assumed almost the same value of 25%.

As it is observed from these results, in order to compare each survival-curve obtained from each irradiated sample, *E. coli* should be irradiated with gamma-ray at a dose-rate exceeding a certain minimum limit when bacteria are suspended in various media. Further, the rich protective substance resulted in a higher dose-rate limit than the poor protective matter in the medium used.

TABLE II. DOSE-RATES AND DURATIONS OF IRRADIATION ON SAMPLES AT THE DOSE OF 15,000 REP IN CASE OF USING A NUTRIENT AGAR MEDIUM CONTAINING 10% HORSE-SERUM

Sample No.	Dose-rate rep/hr	Irradiation Time	Survival	Survival Ratio
—	0	0 hr	583	100%
1	12.618	1 h–11 m	204	35.0
2	9.616	1–34	201	34.5
3	7.571	1–59	194	33.3
4	6.142	2–26	203	34.8
5	5.042	2–59	198	34.0
6	4.230	3–34	200	34.3
7	3.598	4–11	210	36.0
8	3.044	4–56	220	37.7
9	2.443	6–08	238	40.9
10	2.034	7–23	257	44.1

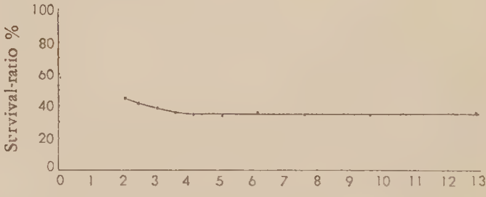

FIG. 3. Relation between Survival-ratio of *E. coli* and Dose-rate of Gramma-ray at the Dose of 15,000 rep in a Nutrient Medium containing 10% Horse-serum.

(2) **Experiment 2:** In Report III, the author has reported that *E. coli* preserved at room temperature in both a nutrient agar medium and a pure agar medium prior to irradiation resulted in higher survival than those irradiated immediately after harvesting and that the longer they were preserved at room temperature, the more bacteria irradiated survived.

In this report, sample containing both *E. coli* and a medium were kept as long as 6 hours in an ice-box prior to gamma-ray irradiation. The media used were a nutrient agar medium and a pure agar medium (non-nutrient). The pre-

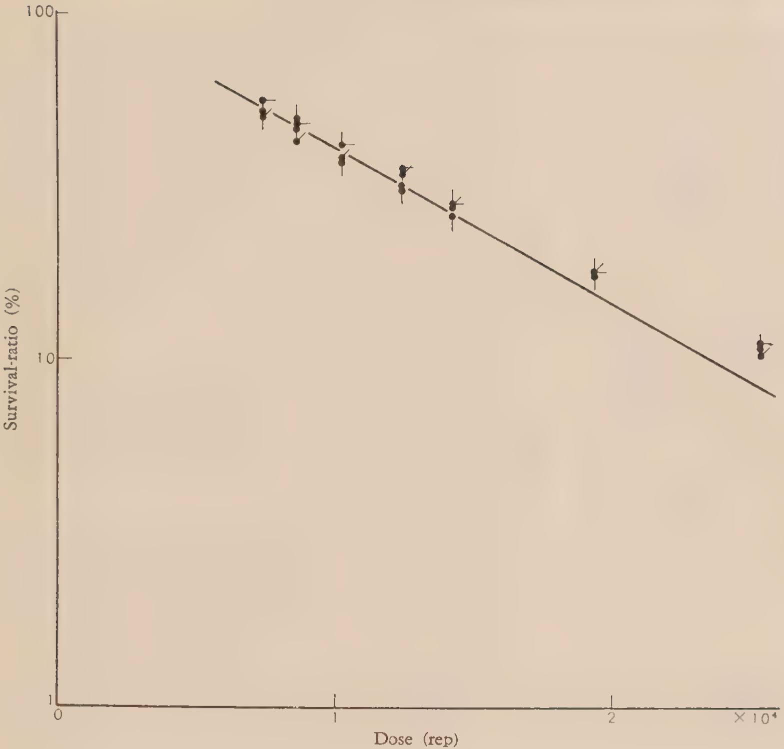

FIG. 4. Survivals of *E. coli* in Broth Agar Medium irradiated by Gamma-ray after Preserving in an Ice-box for Different Period.

● 0 hr 616 cell/ml
● 2 577
● 4 568
● 6 562

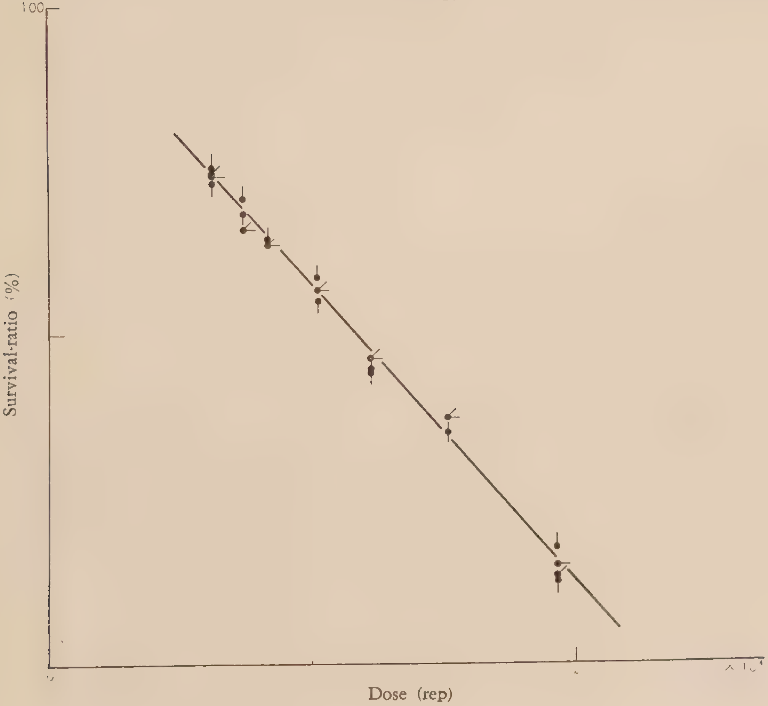

FIG. 5. Survivals of *E. coli* in Pure Agar Medium irradiated by Gamma-ray after Preserving in an Ice-box for Different Period.

● 0 hr 418 cell/ml
● 2 434
● 4 411
● 6 448

servation periods in the ice-box were 2, 4 and 6 hours respectively, the irradiation period being 2 hours in each experiment.

The results of the nutrient agar medium are diagrammed in Figure 4, Figure 5 shows the results obtained with a pure agar medium. As shown in these two figures, no influences of preservation in the ice-box for as long as 6 hours on survivals of the strain in these two media are observed. Therefore, it is supposed that the influences of the preserving procedure reported in Report III should result from biological phenomena. In case of a nutrient agar medium preserved at room temperature, the higher survivals of the strain would be due to the increase in number of viable cells. However, in case of a pure agar preserved at room temperature, the cause of higher survivals is not yet known. Consequently, the following experiment was attempted.

A highly concentrated bacterial suspension in saline was preserved in an incubator at 37°C prior to the preparation of samples. In this case, it was considered that the cells of the strain should become resting. The results are diagrammed in Figure 6. As it can be seen from the figure, survivals of the strain resulted higher than the control, and it was ascertained that gamma-ray resistance of the strain is increased by storage. From this results, the higher survival of *E. coli* preserved at room temperature in a non-nutrient agar may be due to the formation of resting cells resulting from preservation for such a long period.

(3) **Experiment 3:** In Report II, the author has reported that the addition of cells of the same strain which are cells-perished by heat have no influence on the survival-curve of *E. coli*.

In the present experiment, the cells of the strain of *E. coli* sterilized by gamma-ray irradiation were added to the samples. The suspension of perished cells of the strain was prepared by gamma-ray irradiation with cobalt-60 of 40 curies at a distance of 5 cm from the source, for 40 hours. This suspension was added to the living

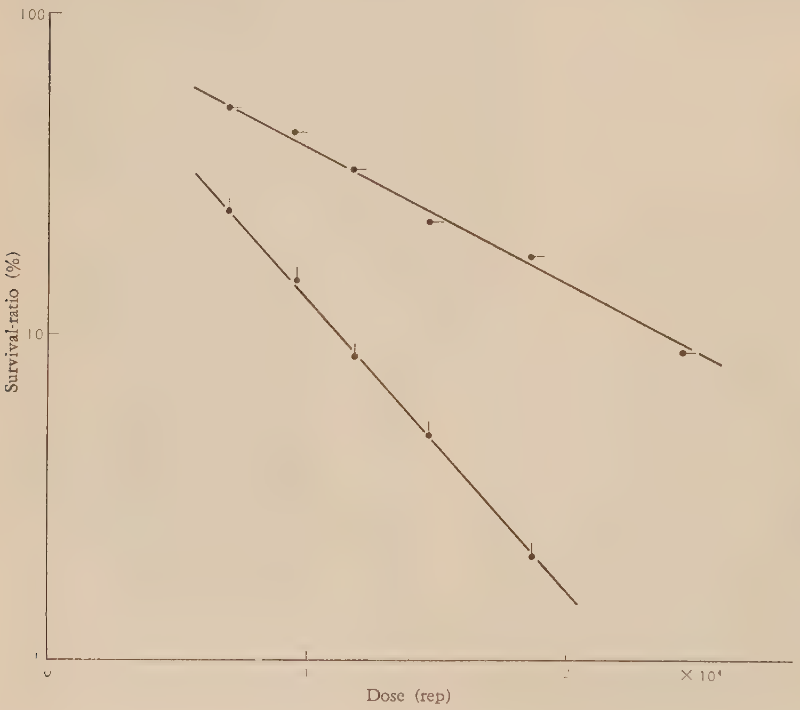

FIG. 6. Influence of Preserving of Bacterial Saline at 37°C Prior to Addition to used Medium upon Survival of *E. coli* irradiated with Gamma-ray.

⫰ 0 hr 418 cell/ml
●– 6 469

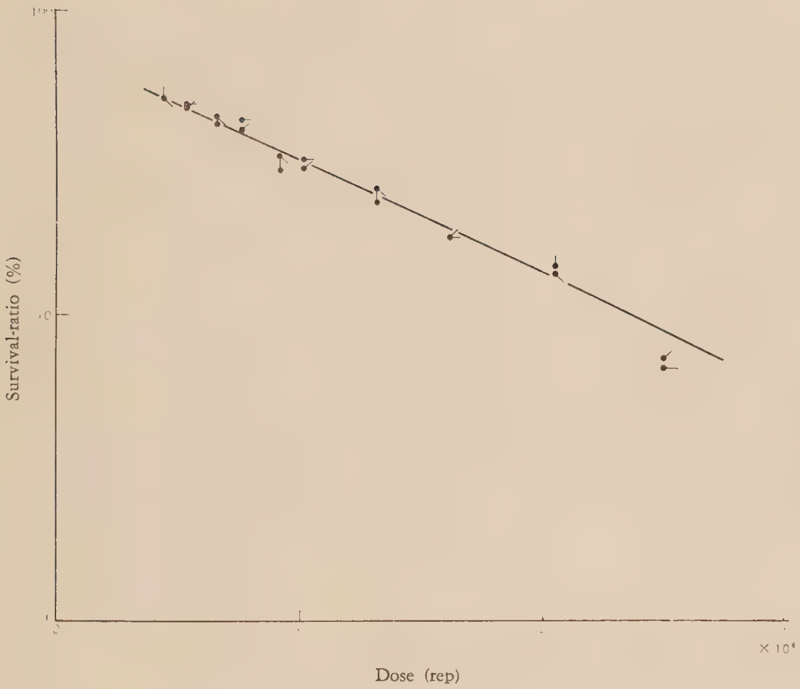

FIG. 7. Influence of Addition of Gamma-ray Sterilized Cells of *E. coli* upon the Same Strain by Gamma-ray Irradiation.

● 0 695 cell/ml
● 10^7 674
●– 10^5 404
● 10^8 375

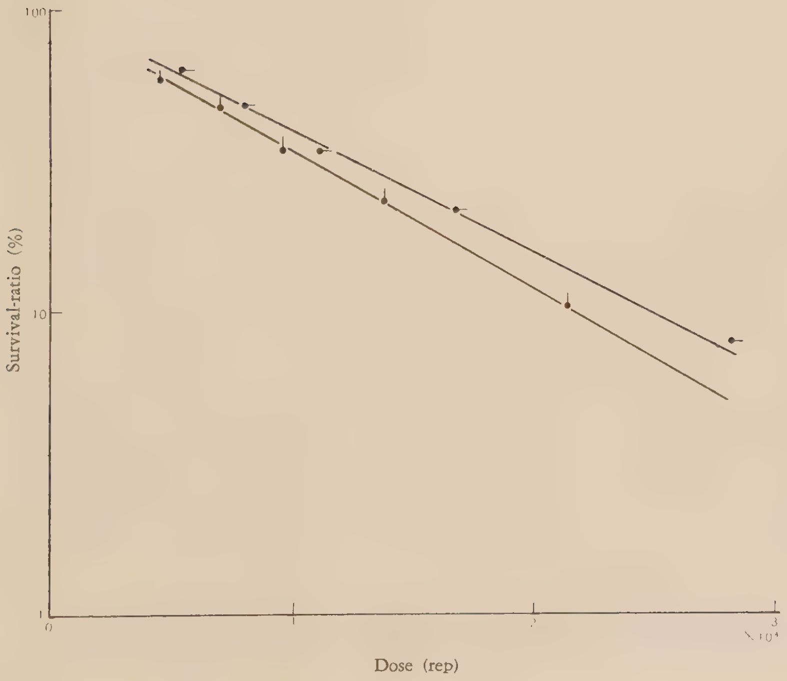

Fig. 8. Influence of Incubating Temperature after Irradiation upon Survivals of *E. coli* irradiated with Gamma-ray.

● 37°C 493 cell/ml
●– 18°C 580

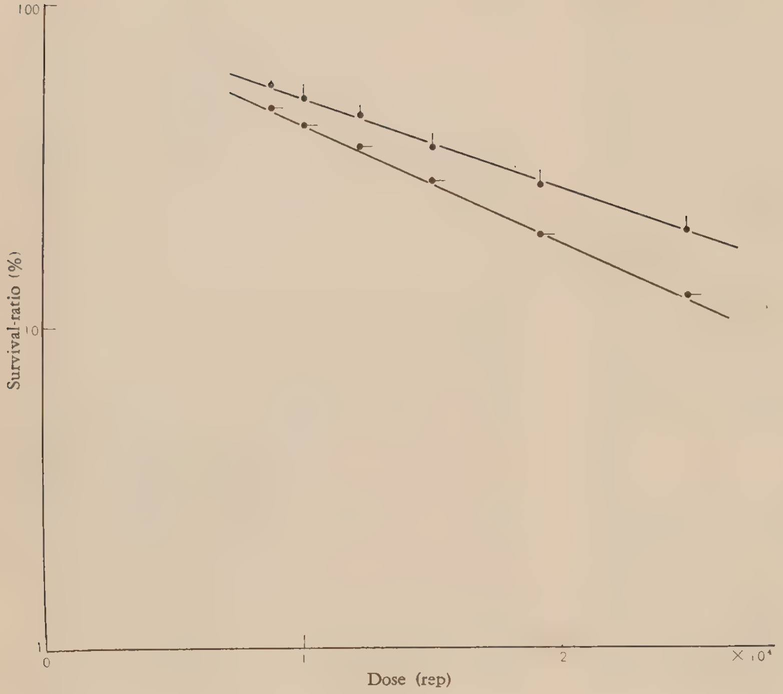

FIG. 9. Influence of Vacuum Procedure upon Survivals of *E. coli* irradiated with Gamma-ray.

•–	controll	683 cell/ml
•|	29 inch vaccum	540

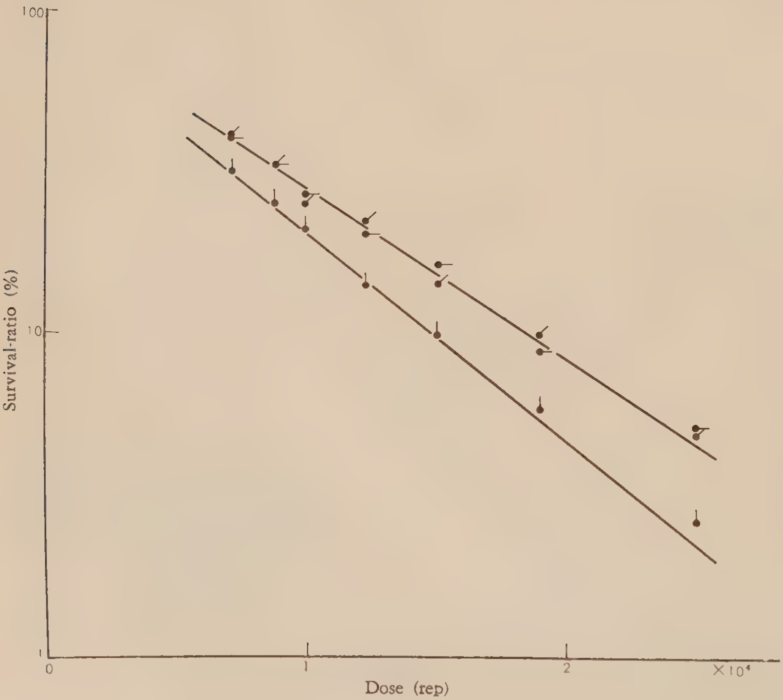

FIG. 10. Influence of Addition of Pereston-N (artificial serum) upon Survivals of *E. coli* contained in a Synthetic Medium by Gamma-ray Irradiation.

•|	0%	601 cell/ml
•/	5	553
•–	10	569

cells at concentrations of 1 : 10, 1 : 10^3 and 1 : 10^5 (living cell count : perished cell count).

The results are diagrammed in Figure 7. As it can be seen from the figure, no influences of the addition of perished cells of the same strain were observed on the survival-curve of *E. coli*.

(4) **Experiment 4:** In Report II, the author has also reported that no influences of incubation on the survival-curve of *E. coli* are observed at either 37° or 25°C.

In the present experiment, bacteria were incubated at 18°C after two hours irradiation until colonies developed. The results are diagrammed in Figure 8. As it can be seen from the figure, the bacteria irradiated and subsequently incubated at 18°C survive better than those incubated at 37°C. Therefore, it would be considered that a part of the irradiated bacteria is recovered from irradiation damage during incubation at 18°C.

(5) **Experiment 5:** In all previous reports, the bacteria were irradiated with gamma-ray in open air. As Goldblith has reported that microorganisms irradiated in vacuum are extremely resistant to gamma-ray[5)], in the present experiment, all samples were packed with a Kureharon film, one of the polyvinylidene-chloride films, under a vacuum value of 29-inch. After irradiation, samples were taken out of the package film and placed into an incubator of 37°C. Therefore, although these samples were irradiated in vacuum, they were incubated in the air.

The results are diagrammed in Figure 9. As seen from the figure, the bacteria irradiated in vacuum survived better than those irradiated in the air. However, the protective effect of the vacuum procedure was not so remarkable as compared with the effects of other protective conditions.

(6) **Experiment 6:** In Report IV, the author has reported on the protective effects of the addition of horse-serum on the medium employed.

In the present experiment, the effect of "artificial-serum" upon the survival of *E. coli* was investigated. This artificial serum was Pereston-N, which contained 6% of polyvinylpyrrolidon (Bayer Corp.).

The used concentrations of the artificial serum in a pure agar medium were 5% and 10%. Figure 10 demonstrates the results from which some protective effects on the survival of the strain are recognized as a result of the addition of the artificial serum to the medium. However, as it can be seen in the figure, the survival-curve obtained from the 10% artificial serum was almost similar to that 5%. Therefore, when an amount of this substance exceeding a certain limit is added, any additive effect upon survival of the strain is not likely to be expected.

SUMMARY

(1) The survival-ratios of *E. coli* irradiated with an equal gamma-ray total dose were almost constant at a dose-rate exceeding a certain limit. This limit differed from each another. In case of nutrient medium containing 10% horse-serum, the limit was 4,000 rep/hr, in case of minimum medium 2,000 rep/hr, and in non-nutrient medium 1,700 rep/hr.

(2) Also, in case of applying the gamma-ray dose-rate below a certain limit, the lower the dose-rate, the more bacteria survived.

(3) When *E. coli* was preserved in an ice-box as long as 6 hours in a nutrient agar or on a pure agar prior to irradiation, no change was observed on the survival of the strain irradiated in either case.

(4) When *E. coli* suspended in saline was preserved in an incubator for 6 hours prior to preparation of the samples, survival of the strain was higher than that of the control. The higher survival is probably due to the formation of resting cells of the bacteria.

(5) When perished cells sterilized with gamma-ray were added to the living cells of the same strain of *E. coli*, no influences on the survival-curve of the living cells were observed.

(6) The survivals of *E. coli* irradiated and

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5) S. A. Goldblith, B. E. Proctor, S. Davison and E.M. Oberle, How Processing Conditions Affect Microorganisms Radioresistance, *Nucleonics*, **13**, 42-45 (1955).

incubated at 18°C were slightly higher than survivals of *E. coli* incubated at 37°C. Some reactivation occurred in incubation of the irradiated bacteria at 18°C.

(7) The survivals obtained from samples irradiated in vacuum were higher than those irradiated in the air.

(8) The protective effects of Pereston-N, which is regarded as an artificial serum, were recognized when *E. coli* was irradiated with gamma-ray.

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Studies on the Reduction of Terpenes with Sodium in Aqueous-ammonia

Part IV. On the Reduction of (+)-Pulegone*

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Received May, 14, 1959

In regard of the reduction of (+)-pulegone with sodium in aqueous-ammonia, application of infra-red analyses and chemical methods revealed the product to be composed of 75% (−)-menthol and 25% (+)-isomenthol.

The reduction of pulegone has been studied by numerous investigators. On catalytic hydrogenation[1,2] at the ordinary temperature, (+)-isomenthone and a small amount of (−)-menthone were obtained. When aluminum- or sodium-amalgam[3] was employed, pinacone was formed. By the used of aluminum-isopropoxide[4,5] the reduction was accompanied by dehydration and *p*-menthadiene was prepared, while pulegol was obtained by lithium-aluminum-hydride[6]. When pulegone was reduced with sodium and alcohol[7,8,9], (−)-menthol and some pulegol were

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[*] For Part III see This Bulletin, **23**,524 (1959).

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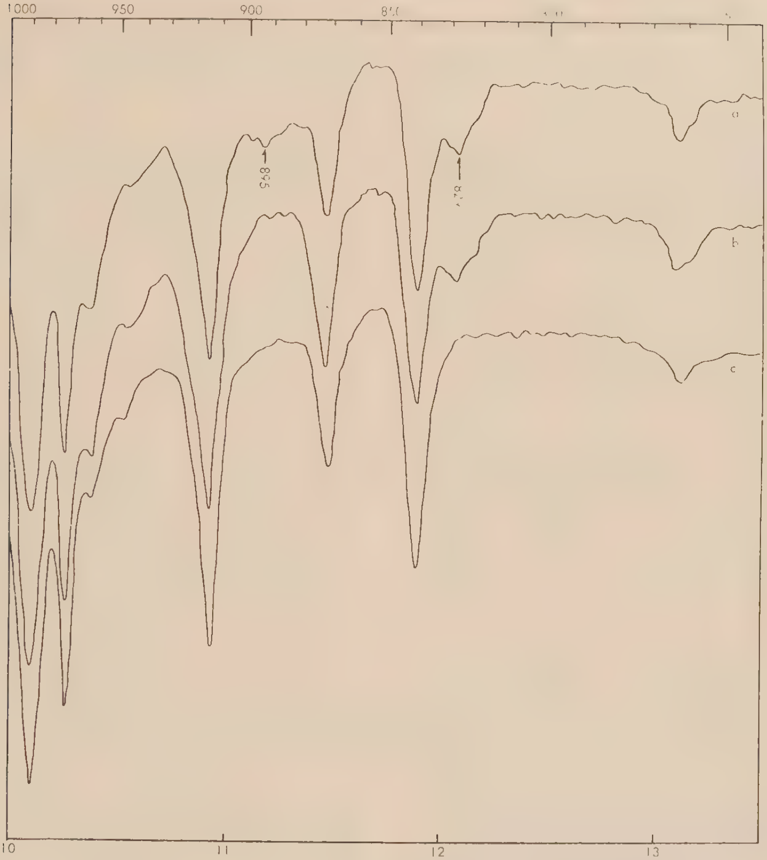

FIG. 1. Infrared Absorption Spectra of Menthol-isomers

(2% carbondisulfide solution)

(a) menthol mixture, (b) mixture of menthol (73%) and isomenthol (27%), (c) menthol

obtained; however much remains to be studied in concern of the contents of isomeric menthol.

In the previous papers, it has been shown that (−)-menthol[10] and (−)-dihydrocarveol[11] are obtained almost quantitatively from (−)-menthone and (−)-carvone respectively by treatment with sodium in aqueous-ammonia. Both reduction products are of the same conformation (e, e, e).

The present study was undertaken in order to determine the composition of isomeric menthol in the reduction product of (+)-pulegone with sodium in aqueous-ammonia. The resulted product was shown to consist of alcohols (96%) and carbonyl compounds (4%). The application of a combination of infra-red analyses and chemical methods revealed that the alcohol fraction consists of (−)-menthol (e, e, e) (75 %) and (+)-isomenthol (a, e, e) (25 %). From the carbonyl fraction separated as semicarbazone, (−)-menthone was confirmed.

EXPERIMENTAL

All melting and boiling points were uncorrected. Ultra-violet absorption spectra were determined in methanol employing a Beckman Model DU-quarz spectrophotometer. Infra-red spectra were recoreded using a

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[10] H. Ueda, Y. Shibahara and S. Shimizu, This Bulletin **23**, 376 (1959).

[11] H. Ueda and S. Shimizu, This Bulletin **23**, 524 (1959).

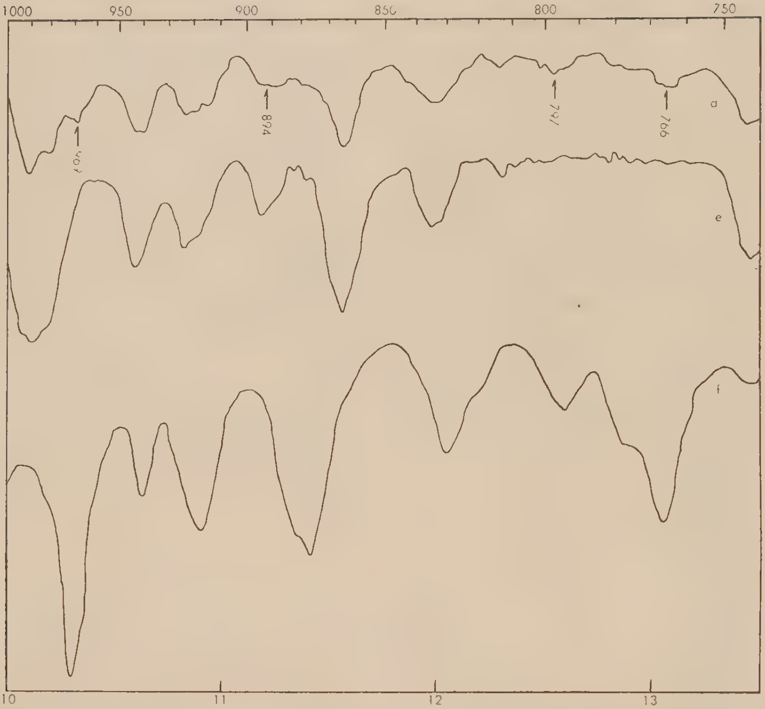

FIG. 2. Infrared Absorption Spectra of Menthone-isomers (in oil)

(d) menthone mixture, (e) menthone, (f) isomenthone

Shimadzu double-beam spectrophotometer at Prof. Nakajima's Laboratory, University of Kyoto.

(+)-Pulegone From pennyroyal oil (POLAK & SCHWARZ. Co., Ltd.) (20 ml), (+)-pulegone (13.2 g) was separated by treatment with sodium-sulfite; b. p. 72~75°/3 mm, d_{20}^{20} 0.9396, n_D^{20} 1.4893, $[\alpha]_D^{20}+21.7°$ (homog.), MR 46.79 (calcd. 45.72). λ_{max} 253mμ, E_{max} 7500, semicarbazone m.p. 174~174.5°, 2,4-dinitrophenylhydrazone m.p. 147.5~148.5°.

Reduction of (+)-Pulegone (+)-Pulegone (9.5 g) was reduced with sodium (8.4 g) in aqueous-ammonia (20 ml) and benzone (20 ml) according to the procedure described previously.[10] The reaction temperature was maintained between 7° and 10°. The product (8.5 g) was obtained by the usual method; b.p. 69~70°/2 mm, m.p. 30~33°, $[\alpha]_D^{20}-26.35°$ (c 23.08 in methanol), λ_{max} 253 mμ, E_{max} 32. The carbonyl content was 4.0% as menthone by the hydroxylamine method.

Separation of Carbonyl compound The reduction product (7.1 g) was treated as described in the preceding report[12]. The carbonyl compound was separated as semicarbazone (158 mg) with m. p. 150~161°. After 3 recrystallizations from methanol, it yielded pure (−)-menthone-semicarbazone (53 mg), λ_{max} 228 mμ, E_{max} 12900, m.p. 185~186°, undepressed by admixture with an authentic sample[10].

Menthol mixture The alcohol fraction which remained unchanged in the above treatment was distilled to give a menthol mixture (6.5 g, 82.8% from pulegone); b. p. 68~70°/2 mm, m. p. 36~37°, $[\alpha]_D^{18}-28.57°$ (c 33.32 in methanol). From the melting point and optical rotation, this menthol mixture seemed to be composed of about 73% of (−)-menthol and 27% of (+)-isomenthol. The infra-red spectrum (a) showed chief absorption bands which were characteristic for (−)-menthol (spectrum (c)) among four menthol isomers[13,14], and moreover, it closely agreed with the spectrum (b) of a mixed sample prepared in the same proportion as mentioned above, by direct weighting of an authentic (−)-menthol and (+)-isomenthol[12]. A small absorption

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[12] H. Ueda and S. Shimizu, This Bulletin **23**, 380 (1959).

[13] Y. R. Naves and J. Lecomte, *Bull. Soc. Chim. France*, **1955**, 792.

[14] H. Ueda and T. Mitsui, *J. Agr. Chem. Soc. Japan*, **28**, 945 (1954).

TABLE I. MELTING POINTS OF (−)-MENTHOL MIXED WITH (+)-ISOMENTHOL

Isomenthol content	10.4	20.7	27.2	30.3	40.1	49.4	60.0	70.6	80.1	89.3%
m.p. of mixture	40.4	40.8	40.6	40.0	42.5	55.5	61.9	68.5	72.8	77.7°

band at 829 cm^{-1} indicated the presence of (+)-isomenthol, but the band at 895 cm^{-1} might be due to an unidentified compound in the reduction product.

The menthol mixture (1.883 g) was completely converted into 3, 5-dinitrobenzoate (3.899 g), m. p. 141∼143°. After 4 recrystallizations from methanol, a homogeneous fraction (2.073g, 53.2% from crude ester) of (−)-menthyl-3,5-dinitrobenzoate was obtained, m. p. 152.5∼153.5°, undepressed by admixture with an authentic sample[10].

Oxidation of Menthol mixture According to the procedure of Read[15], the resulted menthol mixture (1.986 g) was treated with Beckmann's chromic acid at 50° to obtain a corresponding menthone mixture (1.551 g Yield 79.2 %); b. p. 76∼77°/8.5 mm, d_{20}^{20} 0.8965, n_D^{20} 1.4503, MR 46.26 (calcd. 46.19), $[\alpha]_D^{20}+0.83°$ (homog.). The optical rotation showed that it consists of about 75% of (−)-menthone and 25% of (+)-isomenthone. The infra-red spectrum (d) showed the pesence of chief peaks found in spectrum (e) of (−)-menthone, but small absorption bands at 766, 797 and 969 cm^{-1} and no band at 894 cm^{-1} indicated the presence of (+)-isomenthone (spectrum (f)).

Recrystallization of Menthol mixture The menthol mixture (1.551 g) showed the following successive values of m. p., $[\alpha]_D^{20}$ and yields, when recrystallized successively from nitromethane; 37.7∼38.8°, 1.086 g; 37.5∼38.3°, −23.30° (c 14.76 in methanol), 0.783 g; 39.0∼40.0°, −30.7° (c 3.846 in methanol), 0.200 g. It was found that a mixture of (−)-menthol contaminated with 20∼30% of (+)-isomenthol may be hardly separable in this way, owing to the formation of a mixed crystal, which will be explained according to the melting point curve described as follows. From the first mother liquor, only (−)-menthol was separated by the formation of 3, 5-dinitrobenzoate, but other isomers were not identified.

Melting points of Mixture of (−)-Menthol and (+)-Isomenthol A mixture of (−)-menthol (m. p. 43.6°) and (+)-isomethol (m.p. 82.1°) of various compositions were prepared, as shown in the Table I, and the melting points were observed in the usual way for the resolidified materials.

Acknowledgement The authors are indebted to Prof. S. Takei, Prof. T. Mitsui, Prof. M. Nakajima and Assoc. Prof. T. Hashizume, University of Kyoto for their kind advices and suggestions. They also wish to express their sincere thanks to Prof. M. Nakajima for infra-red spectra, Dr. K. Hayashi for pennyroyal oil and Mr. K. Fujimoto, Miss. Y. Fukumoto for their assistance in carrying out this work.

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[15] J. Read, G. J. Robertson and A. M. R. Cook, *J. Chem. Soc.*, **1927**, 1281.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 1, p. 96~100, 1960]

Specific Liberation of Non Protein Nitrogen from κ-Casein Fraction by Rennin and Pepsin*

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Liberation of non protein nitrogen during the process of rennin coagulation of casein in milk had been reported by Nitschmann et al. In this study it is shown that the non protein nitrogen was derived from κ-casein fraction mainly. Rennin and pepsin liberate the non protein nitrogen from κ-casein specifically, while chymotrypsin liberates it non specifically from both κ- and α_R-caseins.

INTRODUCTION

During the primary reaction of rennin coagulation of casein in milk, the specific liberation of non protein nitrogen (12% trichloracetic acid soluble nitrogen) has been found by Nitschmann et al.[1~3]. This liberation is known to occur both in skim milk and sodium caseinate solution. Though it may not be conclusive whether this liberation is attributed to hydrolysis of peptide bond, it seems to be certain that it is closely connected with the transformation of casein into paracasein.

Waugh and Hippel[4] reported the presence of a micelle stabilizing component in casein complex and designated it as κ-casein. They pointed out that this component was changed into the state of coloidal aggregate by rennin action though the detail was not yet reported.

Carinier[5] obtained κ-casein fraction from α-casein and showed that a larger quantity of non protein nitrogen (NPN) was liberated from κ-casein fraction than from true α-casein fraction. In our previous work[6], the same kind of experiment was carried out using calcium-soluble fraction (crude κ-casein fraction) and calcium-insoluble fraction (α_R-casein[7] fraction) of Warner's α-casein[8].

The difference of crude κ-fraction and α_R-fraction in respect of NPN liberation by rennin was more distinctive than that found by Garinier. A definite amount of NPN was liberated from κ-fraction at comparatively earlier stage of rennin action, while the liberation of NPN from α_R-fraction was only negligible[*1]. Other properties such as electrophoretic mobility[9], ultracentrifugal behaviour[10], turbidity and urea-denaturability[6] were changed in κ-fraction by rennin but not in α_R-casein. Furthermore it was demonstrated that interaction between both fractions in unseparated α-casein was retained even after rennin action[10][*2]. Owing to such an interaction it seems to be difficult to detect the marked change of κ-casein by rennin in unfractionated whole casein or α-casein in calcium free system.

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* Studies on Milk Coagulating Enzymes. XIII.

1) C. Alais, G. Mocquot, H. Nitschmann and P. Zahler, *Helv. Chim. Acta*, **36**, 1955 (1953).

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7) J. Long, Q. van Winkle and I.A. Gould, *J. Dairy Sci.*, **41**, 317 (1958).

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9) T. Tsugo and K. Yamauchi, *J. Agr. Chem. Soc. Japan*, **33**, 35 (1959).

10) K. Yamauchi and T. Tsugo, *J. Agr. Chem. Soc. Japan*, **33**, 797 (1959).

[*1] It has been known recently that similar study had been reported by Wake (R. G. Wake, *Australian J. Sci.*, **20**, 147, 1957, through Chem. Abst., 52, 6658, 1958).

Long et al.[7] have reported recently that crude κ-casein consisted of pure κ-casein as a major component and λ-casein as a minor component. As κ-casein fraction used in the previous works contained a slow sedimenting component (λ-casein)[10], the experiment concerning NPN liberation was repeated further using purified κ-casein. Other enzymes were also compared in this respect.

EXPERIMENTAL

(1) Materials and methods

Separation of α_R-, κ- and λ-caseins: Separation of α_R-casein and crude κ-casein has already been reported[9]. It was essentially the same as the procedure of Long et al.[7] except for additional treatment of α-casein at higher alkalinity prior to the addition of calcium chloride. The Warner's α-casein solution was brought to about pH 11.0 so as to be solubilized in a monomeric state, though this treatment seems to be not necessarilly indispensable. After standing it about forty five minutes at 2°C, it was neutralized to pH 7.0 with 1N hydrochloric acid and dialyzed against 0.085 M sodium chloride solution. The dialyzate (protein concentration was 2～3%) was made to 0.25 M of calcium chloride concentration by the dropwise addition of 2 M calcium chloride at pH 7.0～6.6 and allowed to stand overnight. The precipitate (α_R-fraction) was separated by centrifuging at 2,000 r.p.m. for forty minutes and washed with 0.25 M calcium chloride. The supernatant was again centrifuged at 28,000 r.p.m. for sixty minutes in a Spinco Model L Centrifuge (No. 30 Rotor) and clear supernatant solution (crude κ-casein fraction) was collected. It was dialyzed against distilled water repeatedly and finally against 0.085M sodium chloride solution. Potassium oxalate treatment was omitted because it accompanied the co-precipitation of considerable amounts of κ-casein (about one half of total nitrogen in crude κ-casein fraction) as pointed out by Long et al.[7] α_R-Casein was suspended in an adequate amount of water and dissolved by adding about 0.15M of solid potassium oxalate. 0.4 M concentration of potassium oxalate was not adequate as it accompanied the co-precipitation of some of α_R-casein as in the case of κ-casein fraction. The solubilized fraction of α_R-casein was repeatedly centrifuged to remove calcium oxalate and dialyzed against distilled water and then against 0.085M sodium chloride solution. Crude κ-casein fraction was made to pH 7.0 and to 0.2M with respect to sodium chloride, and then centrifuged at 40,000 r.p.m. in a Spinco Model L Centrifuge (No. 40 Rotor) for five hours at low temperature. Upper half of the supernatant (crude λ-casein fraction; sup. B, about 48 ml), lower half of the supernatant (sup. C, about 29 ml) and the viscous precipitate (κ-casein fraction) were separated. About 0.5 ml of top portion (sup. A) in each tube was collected from several tubes immediatly after centrifugation. Crude λ-fraction was concentrated through the celophane membrane the outer side of which was filled with carbowax 1,500[11]. α_R-, purified κ- and crude λ-fractions were adjusted to pH 6.6 with a small amount of 1N sodium hydroxide after dialysis against 0.085M sodium chloride.

Enzymes: Referring to the method of Berridge[12], rennin was purified by electrophoretic technique using Tiselius apparatus (Hitachi A Type Apparatus was used) from the extracted solution of Hansen's rennet tablets after three times salting-out with sodium chloride at pH 5.5. The purified fraction was once crystallized at pH 5.2 and lyophilized. Crystalline pepsin was a commercial product (Nutritional Biochemical Corporation) and crystalline chymotrypsin was kindly given by Miss M. Kondo, Institute of Applied Microbiology, University of Tokyo. Concentration of enzyme solutions was about 0.02 mg/ml for rennin, 0.5 mg/ml for pepsin and 0.67 mg/ml for chymotrypsin respectively.

Methods: Protein concentration of each casein fraction was adjusted by dilution with suitable amounts of 0.085M sodium chloride solution. 0.25ml of the enzyme solution* was added to 5 ml of aliquot of each fraction at 35°C and after a given time 5.25 ml of 24% trichloracetic acid solution was added. After allowing to stand for about one hour, the mixture was filtered and the nitrogen in the filtrate was determined by micro Kjeldahl method. The development of turbidity was traced by measuring the transmission of the reaction mixture with Coleman's Nephelometer using no filter. Phosphorus content of each fraction was analyzed by colorimetric method after wet digestion with sulfuric acid and hydrogen peroxide[13].

[*2] This view seems to agree with the recent study of Waugh and Gillespie (D. F. Waugh and J. M. Gillespie, Dairy Sci. Abst. 20, 1032, 1958).

11) K. Arai, Y. Sakaguchi and K. Nomiyama, *The physico-Chemical Biology, Japan*, **3**, 56 (1956).

12) N.J. Berridge, *Milchwissenschaft*, **10**, 195 (1955).

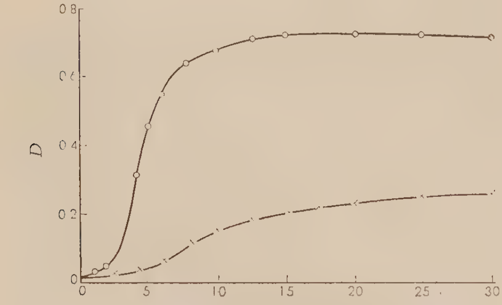
* With this amount of the enzymes 5 ml of whole milk (pH 6.6) was coagulated in about six minutes by rennin, in about four minutes by pepsin and in about 7.5 minutes by chymotrypsin respectively.

(2) Phosphorus-to-nitrogen ratio of each fraction Phosphorus-to-nitrogen (P/N) ratio of all fractions described above is compared in Table I. Phosphorus-to-nitrogen ratio of α_R-fraction was more than twice of that of κ-casein and almost equal to that of the top portion, which was considered to be pure λ-casein. As phosphorus-to-nitrogen ratio of the upper half portion (crude λ-casein) was considerably less than that of the top portion, it was most likely that this fraction was mixed with κ-casein. The percentage of phosphorus in Table I is not a true value but only a calculated value on the assumption that protein concentration is equal to N×6.38 for all fraction. The difference between phosphorus % of κ-casein in Table I and that reported by Long et al.[7] (<0.33%) is probably not a true discrepancy. Conversion factor from nitrogen to protein concentration may be considerably higher than 6.38 in the case of κ-casein.

TABLE I. P/N RATIO OF EACH FRACTION OF α-CASEIN

Fraction	P/N	P%*
α_R	0.0746	1.17
Crude κ	0.0323	0.51
κ (Precipit.)	0.0280	0.44
λ (Sup. A; Top portion)	0.0727	1.14
Crude λ (Sup. B; Upper half)	0.0567	0.89
Sup. C (Lower half)	0.0420	0.66

* Calculated on the assumption that protein concentration is equal to N×6.38.



Reaction time in minutes

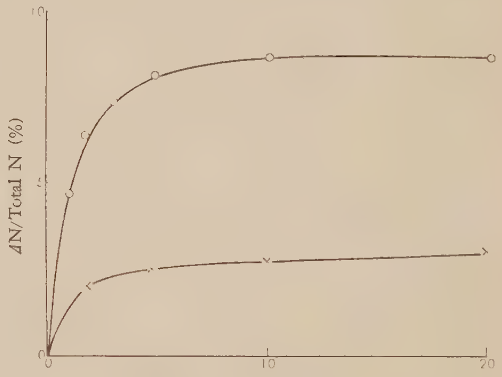
FIG. 1. Change of Transmission of κ- and Crude λ-Fractions by Rennin.

○—○ κ-fraction (Protein concentration: 0.4%)
×—× crude λ-fraction (〃 : 〃)
D: optical density

(3) Development of turbidity in κ- and crude λ-fractions by rennin Pertinent result is shown in Fig. 1. The development of turbidity was marked in κ-fraction. Though turbidity is developed in crude λ-fraction to some extent, it is doubtful whether it is attributed to the change of λ-casein itself.

(4) Liberation of NPN from κ- and crude λ-fractions by rennin The leberation of NPN from κ- and crude λ-fractions is as illustrated in Fig. 2. The liberation of NPN from α_R-casein was omitted as it had been already reported[6]. It was only within 0.6% of the total nitrogen in α_R-casein even after sixty minutes. The curve of purified κ-casein in Fig. 2 was identical with that of crude κ-casein except that in purified κ-casein slightly larger amount of NPN was liberated.

(5) Comparison of rennin with pepsin and chymotrypsin The liberation of NPN from α_R-fraction and κ-fraction by pepsin and chymotrypsin is shown in Fig. 3. The liberation curve of pepsin was found to resemble to that of rennin. Only negligible NPN was liberated from α_R-fraction by pepsin as well as by rennin. Chymotrypsin showed different types of curves both for α_R- and κ-fractions.



Reaction time in minutes

FIG. 2. Liberation of Non Protein Nitrogen (NPN) from κ- and Crude λ-Fractions by Rennin.

ΔN Increase of non protein nitrogen (NPNt−NPNo)
○—○ κ-fraction (protein concentration 0.4% NPNo/Total N 4.90%)
×—× λ-fraction (protein concentration 0.2% NPNo/Total N 22.2%)

DISCUSSION

Crude λ-fraction used in the experiments of Fig. 1 and Fig. 2 contained considerable amounts

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[13] M. Nakamura, *J. Agr. Chem. Soc. Japan*, **24**, 1 (1950).

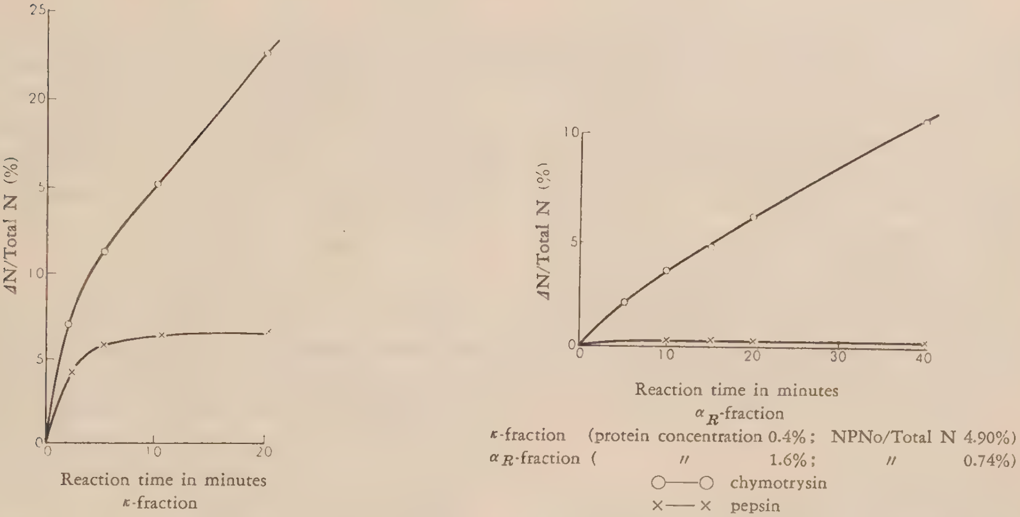

FIG. 3. Liberation of Non Protein Nitrogen from α_R- and κ-Fractions by Pepsin and Chymotrypsin.

of κ-casein as mentioned above. During separation after centrifugation, dispersion or disturbance of both fractions might occur. According to this procedure it seemed difficult to obtain pure λ-casein without considerable loss. As crude λ-fraction probably contained κ-casein, it was uncertain whether NPN was liberated from pure λ-casein by rennin. Experiments could not be carried out on the top portion because of its low yield. If phosphorus to nitrogen ratio of pure λ-casein is 0.0727 and that of pure κ-casein is 0.028 (Table I), the amount of κ-casein contained in crude λ-fraction (its phosphorus to nitrogen ratio is 0.0567) is calculated to be about one third of the latter*. Since the amount of NPN liberated from crude λ-fraction was about one third of that from purified κ-fraction, it is most likely that NPN from the former was liberated from κ-casein contained in it. The development of turbidity in crude λ-fraction by rennin (Fig. 1) was also probably due to κ-casein in this fraction as turbidity was not detected when rennin was added to an aliquot of the top portion. NPN was liberated from κ-casein by rennin rapidly as the initial stage of reaction until reaching maximum value. Pepsin closely resembled to rennin in the shape of curve though the amount of NPN liberated by pepsin was slightly less than that by rennin. Liberation of NPN from α_R-casein by rennin and pepsin was only negligible. Liberation of NPN from κ-casein by chymotrypsin was markedly different from that by rennin or pepsin. The shape of this curve may represent general proteolysis. NPN was markedly liberated also from α_R-casein by chymotrypsin.

Comparison of yield and some properties of curds coagulated by various enzymes including rennin, pepsin and chymotrypsin has been reported by the authors[14]. Considerable re-solution of curd occurred in coagulation by proteases other than rennin or pepsin, while in rennin or pepsin coagulation the yield of curd was highest and re-solution did not occur. Though the change of κ-casein may be essential for the coagulation of milk by all kinds of milk coagulating enzymes, general proteolysis will occur not only in κ-casein but also in other casein components when milk is coagulated by some enzymes other

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* If κ-casein content in crude λ-fraction is $x\%$, x will be calculated as follows:

$$0.0727\,(100-x)+0.028\,x=0.0567\times100$$
$$x=36\ \%$$

[14] T. Tsugo and K. Yamauchi, Proc. XVth Int. Dairy Congr. **2**, 643, London, 1959.

than rennin or pepsin. On the contrary, rennin and pepsin are considered to have specific action on κ-casein. They will break down the structural configuration of the molecule of this component specifically accompanying the liberation of the definite amounts of NPN.

The liberation of NPN from κ-casein by rennin is assumed to be a first order reaction same as described in the report by Nitschmann et al.[3] using unfractionated casein. Furthermore, transmission curves is also possible to be expressed as a first order type reaction if log $\left(\frac{D_\infty - D_t}{D_\infty}\times 100\right)$ (D_∞: opticol density at maximum; D_t: optical density at t minutes after rennin addition) is plotted against time, though in this case induction period at initial stage is not fitted on this line. Fig. 4 was obtained by this manner from the curves in respect of κ-casein in Fig. 1 and Fig. 2.

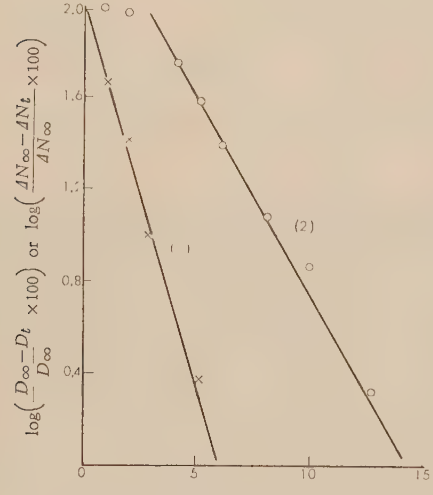

FIG. 4. Linear Expression of the Transmission Curve and NPN Liberation Curve of κ-Fraction.

(1) Liberation of NPN
(2) Transmission

SUMMARY

κ-Casein and crude λ-casein were separated from crude κ-casein (calcium soluble fraction of Warner's α-casein). Development of turbidity and liberation of non protein nitrogen (NPN) in crude κ-casein by rennin, as already reported, was assumed to be attributed to the change of κ-casein itself.

Liberation of NPN from α_R-casein (calcium insoluble fraction of Warner's α-casein) and purified κ-casein by pepsin and chymotrypsin was compared with that by rennin. It was demonstrated that pepsin, as well as rennin, liberated a definite amount of NPN from κ-casein and scarcely liberated it from α_R-casein, while chymotrypsin liberated NPN from both fractions increasingly with the increment of reaction time.

Acknowledgements The authors wish to express their sincerest thanks to Prof. Rinjiro Sasaki, Department of Agricultural Chemistry, University of Tokyo, for his kind guidance throughout this work.

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On the Urea Denaturation of Paracasein*

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Visible difference of casein and paracasein in calcium free solution is not ordinarily detected. Marked difference is found, however, if they were previously treated with urea. Paracasein becomes partially precipitable when it is dialyzed against calcium free buffer or water after urea treatment. By the same treatment casein is not denatured. It is considered that some component of casein complex is transformed into the structure, which is easily denatured by urea, after the action of rennin.

INTRODUCTION

It is well known that casein forms comparatively stable micelle by calcium chloride while paracasein forms coagulum.

In spite of such a marked change of casein by rennin, it is difficult to detect the difference between casein and paracasein in calcium free solution except for slight change of some properties such as electrophoretic[1] or ultracentrifugal[2] behaviors. In this report it is presented, however, that the difference between casein and paracasein can be visibly detected when both are treated with urea and dialyzed against calcium free buffer. It may support the view that rennin changes some component composing casein complex specifically.

EXPERIMENTALS

(1) **Materials and methods** Unfractionated casein was precipitated by the addition of 1N hydrochloric acid till pH 4.5～4.6 at room temperature. After washing with water, it was dissolved with diluted sodium hydroxide at pH 6～7 and again precipitated. The precipitate was dried after washing with water, alcohol and ether. α-Casein was purified by Warner's method[3] and also β-casein by the urea method of Hipp et al.[4]. Crystalline rennin was provided by Dr. M. Oeda. Electrophoresis was carried out in phosphate-sodium chloride buffer consisting of 0.00632 M sodium hydrogen phosphate, 0.00105 M potassium dihydrogen phosphate and 0.08 M sodium chloride (pH 7.3, ionic strength 0.1). Hitachi A-Type Tiselius Apparatus was used (marco cell ; cross-sectional area 0.75 cm^2). Patterns were photographed after 80 minutes electrophoresis with a constant current of 18 mA. Nitrogen was determined by micro Kjeldahl method.

(2) **Influence of urea on casein** Eight grams of urea were added to 10 ml of 3% casein solution dissolved in sodium hydroxide (pH 6.6) and the volume was adjusted to 20 ml with water. After six lots of urea-containing samples were left standing at 2°, 20° and 37°C for one or two overnights, they were dialyzed against phosphate-sodium chloride buffer of pH 7.3 described above repeatedly at low temperature (2°～4°C) until urea was completely removed. Neither opalescene nor precipitate was detected in these dialyzates. One ml of 0.1 M calcium chloride was added to 5 ml of each dialyzate. Stable micelles were formed and so in urea-untreated sample as well. Electrophoresis was carried out on this urea-treated sample. No difference was found between urea-treated and untreated caseins.

(3) **Influence of urea on paracasein** 0.4 ml of rennin solution (0.04 mg rennin) was added to 10 ml of sodium caseinate solution described above and the mixture was left for one hour at 35°C. Then urea was immediately added and the volume was adjusted to 20 ml. After standing for about twenty hours at low temperature, sample was dialyzed against the same buffer used in (2). After dialysis the appearance of precipitate

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* Studies on Milk Coagulating Enzymes. XIV.

[1] H. Nitschmann and W. Lehmann, *Experientia*, **3**, 153 (1947).

[2] H. Lück and F. J. Joubert, *Milchwissenschaft*, **11**, 238 (1956).

[3] R. C. Warner, *J. Am. Chem. Soc.*, **66**, 1725 (1944).

[4] N. J. Hipp, M. L. Groves, J.H. Custer and T. L. McMeekin, *J. Dairy Sci.*, **35**, 272 (1952).

TABLE I. INFLUENCE OF UREA TREATMENT ON CASEIN AND PARACASEIN

Experimental No.	Treatment	Formation of precipitate	Coagulation by $CaCl_2$
1	Urea untreated casein	−	− *1
	Urea treated casein	−	− *1
	Urea untreated paracasein	−	+
	Urea treated paracasein	+	+
2	Urea treated α-casein	−	− *1
	Urea treated para α-casein	+	+
	Urea treated β-casein	−	− *2
	Urea treated para β-casein	−	− *2
3	Urea treated casein (Acid casein from the same milk below)	−	− *1
	Urea treated paracasein (Separated from curd of skim milk directly)	+	+

*1) White opalescent solution.
*2) Coagulated by warming above about 20°C.

was observed and the coagulability of the dialyzate by calcium chloride was tested. The same experiment was carried out on the fractionated α- and β-caseins and also on the paracasein which was separated directly from rennin curd (coagulated skim milk) by centrifugation and washing. The result is presented in Table I. The precipitate appeared in both paracaseins from sodium caseinate solution and from curd of skim milk. As precipitate consists of small particles, shaking causes marked opalescence and it takes a considerable long time to fall off. As this precipitate apparently appeared in para α-caseinate but not in para β-caseinate, it seems to be originated from α-casein fraction of paracasein mainly.

The same dialyzate of urea-treated paracasein was prepared and centrifuged at 2,000 r. p. m. for fifteen minutes. The supernatant was decanted carefully and examined by electrophoresis. The result is shown in Fig. 1. Relative concentration of α-casein slightly decreased and that of β-casein increased contrary to the supernatant of urea-treated paracasein compared with urea-untreated whole fraction of paracasein or casein. This means probably that some portion of para α-casein was removed as the precipitate. Furthermore, it could be seen that the electrophoretic heterogeneity of para α-casein peak, as reported by Nitshmann and Lehmann[1)], became undetectable after complete centrifugation of the urea-treated paracasein. Fig. 1-d was the same sample as Fig. 1-c except that it was somewhat opalescent as centrifugation was not completed (few minutes at 2,000 r.p.m.). In this sample the electrophoretic heterogeneity around α-peak could be observed slightly. It was impossible to obtain an electrophoretic pattern of the urea-treated paracasein without removing the precipitate to some extent owing to its marked opalescence.

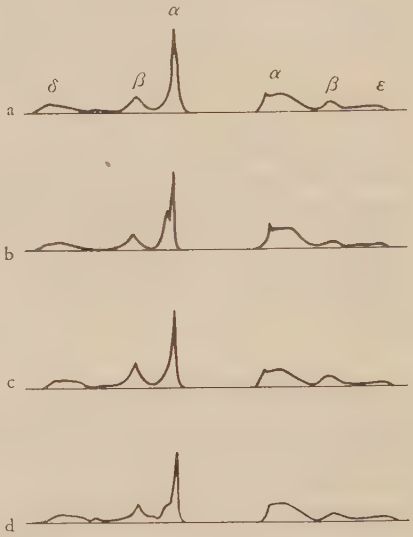

FIG. 1. Electrophoretic Patterns of Paracasein treated or untreated with Urea

a. Whole fraction of urea treated acid casein
b. Whole fraction of urea untreated paracasein
c. Supernatant fraction of urea treated paracasein (2,000 r.p.m. 15 minutes)
d. Supernatant fraction of urea treated paracasein (2,000 r.p.m. 3~4 minutes)

Phosphate-NaCl buffer (pH 7.3, $\mu=0.1$); protein concentration, about 1%; temperature, about 6°C

(4) Influence of temperature and time of urea treatment on the amount of the precipitate Sodium caseinate and sodium paracaseinate solutions were prepared in the same manner as in (3). Urea was added to 6.66 M (protein concentration was about 1.5%) and was left standing at 35° or 2°C. After different times, a portion was dialyzed against cold tap water for one day and against the buffer of pH 7.3 used in (2) repeatedly further for another day. The dialyzate was centrifuged at 2,000 r.p.m. for fifteen minutes. The amount of precipitated protein was calculated from the difference between nitrogen of the supernatant and total nitrogen of the suspension before centrifugation. The result is presented in Table II. Heated sample described in Table II was the same as unheated one except that the former was heated at 80°C for ten minutes after rennin action, followed by the addition of urea. It is apparent that the amounts of precipitate will not necessarily increase by increasing the time or raising the temperature of urea treatment, though exact quantitative results probably cannot be expected in such a kind of procedure because of the difficulty involved in removing urea in a short time by dialysis, and furthermore, it entails a comparatively high degree of error to calculate the amount of the precipitate when it is slight. Among the conditions used in Table II, the highest amount of precipitate was obtained by urea treatment at 2°C for twenty four hours. The precipitation of paracasein by urea denaturation seems to be a considerably complicated phenomenon. When the turbid sample after dialysis was warmed to 35°C, the precipitate disappeared and a clear solution was obtained after several minutes. It is assumed that such a disappearance of precipitate was due to interaction between the precipitated protein and the supernatant protein, as the disappearance of precipitate did not occur when the precipitate was warmed separately after suspending in the buffer.

TABLE II. INFLUENCE OF TIME AND TEMPERATURE OF UREA TREATMENT AND HEATING PREVIOUS TO UREA TREATMENT ON THE DENATURATION OF PARACASEIN BY UREA

Time* of urea treatment (hours)	Temperature of urea treatment	Precipited N/Total N (%)			
		Heated paracasein		Unheated paracasein	
		35°C	2°C	35°C	2°C
0.25		5.7	—	5.5	—
1		6.3	1.5	6.6	—
24		2.3	9.0	—	—
48		0.6	5.7	0.2	5.3

* Time intervals from addition of urea till beginning of dialysis.

(5) **Phosphorus-to-nitrogen ratio of the precipitate** The dialyzate of paracasein taeated with urea of twenty four hours at 2°C was centrifuged and the precipitate was washed with cold water. It was dispersed in a small amount of water and dialyzed against distilled water to remove phosphate ion completely. As the precipitate became grosser coagulant after dialysis, a small amount of 0.1N sodium hydroxide was added till homogeneous suspension was obtained. Phosphorus and nitrogen content of this suspension was analyzed and phosphorus-to-nitrogen ratio was calculated. Phosphorus was analyzed by modified Allen's method[5]. The result is shown in Table III including phosphorus-to-nitrogen ratio of separated fractions of casein or paracasein reported in literature. Though the precipitate seemed to be originated from α-casein mainly, its phosphorus-to-nitrogen ratio was found to be considerably lower than that of α-casein and even somewhat lower than that of β-casein.

TABLE III. COMPARISON OF P/N RATIO OF UREA DENATURED FRACTION OF PARACASEIN AND P/N RATIO OF OTHER CASEIN COMPONENTS

Fraction	P/N	P%	Literature
Urea denatured fraction of paracasein	0.0326	0.511*	
α-casein	0.0631	0.982	(3)
β-casein	0.0391	0.602	(3)
para α_1-casein	—	1.6	(6)
para α_2-casein	—	0.85	(6)
κ-casein	0.0280	0.44*	(7)

* Calculated on the assumption that protein concentration is equal to N×6.38.

DISCUSSION

From the above experiments it is quite apparent that casein contains some component which becomes denaturable by urea treatment after rennin action, though further investigation is required on the property or composition of the denatured protein itself*. It may be interesting to note that some component becomes urea denaturable after rennin action, though rennin action itself may not be a denaturing action. The precipitate seems to be probably originated from α-casein mainly and to be closely connected with κ-casein. In the previous report[8] the authors have shown that calcium soluble fraction of Warner's α-casein, the main component of which is known to be κ-casein designated by Waugh et al.[9] became urea-denaturable after rennin action. When para κ-fraction was treated with 6.66 M urea at 2°C for twenty four hours and dialyzed in the same way as described in experiment (3), it was found that about one half of the total nitrogen in this fraction became precipitable. In urea-untreated para κ-casein, though colloidal aggregates were formed in it, no precipitate was removed by ordinary centrifugation. Furthermore, in rennin untreated κ-casein and α_R-casein treated or untreated with rennin, precipitation was not detected after urea treatment. From these experiments and low phosphorus-to-nitrogen ratio of the precipitate comparable with that of κ-casein, it is most probable that the precipitated fraction of paracasein treated with urea was mainly originated from κ-casein or at least related with it. Urea probably splits the casein complex into each component as monomeric state(10). In paracasein, α_R- and para κ-casein complex will also be splitted into α_R- and para κ-caseins and the latter will be denatured at the same time. If urea is removed by dialysis at low temperature from such a system, denatured para κ-casein will aggregate each other and precipitate appears earlier than the reformation of complex with α_R-casein occurs. When the temperature of this mixture containing the precipitate is raised, denatured para κ-casein will interact with α_R-casein and subsequently the precipitate will disappear. It has been reported previously(11) that α_R-casein and para κ-casein (urea untreated) interact irreversibly by rising temperature. Such a characteristic may be retained after urea denaturation of para κ-casein. It may be assumed that κ-casein transforms into the structure which can be easily denatured by urea, accompanying the liberation of non protein nitrogen from some portion in molecule which may have an important role for its configuration, though rennin action itself may not be a denaturing action.

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[7] T. Tsugo and K. Yamauchi, This Bulletin, **24**, 93 (1960).

[8] K. Yamauchi and T. Tsugo, *J. Agr. Chem. Soc. Japan*, **33**, 38 (1959).

[9] D. F. Waugh and P. H. von Hippel, *J. Am. Chem. Soc.* **78**, 4576 (1956).

* After finishing of this work it was known that the denatured protein (precipitate) can be dissolved at higher pH. It was observed that the dissolved portion of this precipitate consisted of two main components which had slightly different mobilities from that of α- and β-caseins respectively by means of electrophoresis in phosphate-NaCl buffer of pH 11.3 (μ=0.1).

SUMMARY

Paracasein was found to differ from casein markedly in its behaviour to urea. The former is partially denatured by urea. Precipitate appears in paracasein which is dialyzed against calcium free buffer after treatment with 6.66 M urea at low temperature. It is assumed that this precipitate may have mainly originated from para α-casein and possibly be related to para κ-casein.

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Radioactive Contamination of Milk and Milk Products in Japan

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Radioactive contamination of milk and milk products in Japan was studied extensively. Samples were collected monthly or every season from several districts of Japan.

In this report, total radioactivity of raw milk and skim milk powder and strontium-90 (Sr-90) concentration of skim milk powder and sweetened condensed milk were measured. These experiments show that there is neither seasonal variation nor regional variation in total radioactivity, and that in the measurements of Sr-90 concentration the values in northern districts are higher than those in other parts of Japan.

INTRODUCTION

On account of nuclear fission products born out of nuclear weapon tests, there is an increasing tendency in the radioactive contamination upon air, rain water, soil, cereals, vegetables, farm animals and their products from year to year.

Among nuclear fission products, Sr-90 and cesium-137 (Cs-137) which have long half-lives are said to be the most dangerous elements for human body. Sr-90, as well as calcium, belongs to the alkaline-earth metals, and, therefore, its chemical properties resemble to those of calcium. In this respect, it is reasonable to expect that strontium behaves similarly as calcium in biological processes when it enters into the human body.

Europeans and Americans usually take their calcium mainly from milk and milk products, while Japanese take chiefly from vegetable foods. But recently the consumption of milk and milk products in Japan has gradually been accelerated year by year. Especially, artificial-feeding infants take their nutrition mainly from milk and milk products. Therefore it is of significance to investigate the radioactive contamination in milk and milk products in Japan.

For this purpose, the measurements of total radioactivity in raw milk and skim milk powder and concentration of Sr-90 in skim milk powder and sweetened condensed milk were carried out accordingly.

EXPERIMENTAL

I. Total radioactivity in raw milk and skim milk powder

(1) Samples

Raw milk and skim milk powder which were collected monthly from June 1956 to May 1957 from 41 and 17 localities respectively were used for radioactivity assay. But occassionally when skim milk powder was not manufactured in some localities, whole milk powder was used for assay instead of skim milk powder.

(2) Measurement of radioactivity

(a) Ashing of samples

Raw milk: 180 ml of raw milk were measured into a beaker, evaporated in boiling water, transferred to a crucible, heated two hours in a crucible type electric furnace until a carbonaceous mass was obtained, and then transferred to a muffle furnace and heated below 550°C until a white ash was obtained.

Skim milk powder: 25 g of skim milk powder (in case of whole milk powder 20 g was used) were weighed out into a crucible, and then treated in the same way as mentioned in the ashing of raw milk.

(b) Measurement of radioactivity: About 0.5 g of ash was weighed out into a counting tray, 25 mm in diameter and 6 mm in depth, manufactured by the Physical and Chemical Research Institute.

After leveling the surface of ash with a glass rod having a flat end, it was weighed accurately, and then the radioactivity was measured with Model 32-C Geiger Müller counter tube manufacture by the Physical and Chemical Research Institute. The radioactivity was expressed as cpm per 1 g of ash. The time required for

TABLE I. TOTAL RADIOACTIVITY OF RAW MILK (cpm/g of Ash)

Locality		Number	1956 June	July	August	September	October	November	December	1957 January	February	May
Hokkaido		A 1	45	48	46	47	48	45	48	36	39	47
〃		B 2		56	40	49	45	44	46	46	54	52
〃		C 3	42	47	45	46	51	45	47	39	50	47
〃		D 4		50	44	51	40	46	48	30	40	49
〃		E 5	54	47	46	43	46	41	45	40	40	39
〃		F 6	41	53	41	53	52	46	44	43	42	45
〃		G 7	44	51	44	36	49	48	50	44	46	48
Tôhoku	Iwate	8	52		51	45	43	53	49	40	38	37
〃	Miyagi	9	51	47	45	46	46	45	47	40	52	52
〃	Yamagata	A 10	45	51	46	46	49	47	49	33	45	39
〃	〃	B 11		49	42	46	46	44	46	41	35	39
〃	Fukushima	12	41	54	45	23	44	48	46	45	46	42
Kanto	Tochigi	13	42	57	42	49	44	49	48	44	44	37
〃	Gunma	14	48	47	46	45		43	45	49	35	40
〃	Ibaragi	15	45	49	41	45	50	48	49	36	36	39
〃	Saitama	16	49	50	42	43	48	45	48	50	40	30
〃	Tôkyo	17	49	43	49	48	43	50	40	43	46	23
〃	Chiba	18	51	57	47	46	49	51	48	60	53	38
〃	Kanagawa	19	45	40	49	43	52	48	50	39	46	42
Chûbu	Niigata	20	51	48	48	46	46		47	52	42	36
〃	Nagano	A 21	45	46	33	48	40	39	40	44	34	41
〃	〃	B 22	48	47	45	43	47	47	46	35	41	40
〃	Ishikawa	23	57	48	45	49	48	45	47	35	46	74
〃	Yamanashi	A 24	45	53	43	44	45		46	41	47	42
〃	〃	B 25	48	47	42	41	37		43	39	50	
〃	Shizuoka	26	52	54	38	44	46	45	46	40	46	50
〃	Aichi	27	49	45	45	45	48	43	45	42	48	52
Kinki	Kyôto	28		47	48	47	49	47	48	44	40	39
〃	Ôsaka	A 29	48		40	42	44	46	45	41	45	
〃	〃	B 30	50	51	43	44	44	45	47	43	45	43
〃	Hyôgo	A 31		47	42	46	45	40	45	44	42	52
—	〃	B 32		51	39	43	46	55	50	51	54	42
Chûgoku	Tottori	33		61	45	44	47	48	46	40	54	51
〃	Okayama	A 34	46	53	42	44	43	49	47	35	53	42
〃	〃	B 35		50	38	43	53		50	25	55	38
〃	Yamaguchi	36	48	47	38	43	46	44	46	48	46	50
Shikoku	Tokushima	37	52		45	47	45	46	46	55	50	49
〃	Ehime	38		49	36	49	55	47	48	40	46	42
Kyûshû	Saga	39	51	46	43	46	47	48	50	35	47	51
〃	Kumamoto	40	54	56	53	46	49	51	50	55	58	60
〃	Kagoshima	41		46	40	41	47		46	44	38	44

TABLE II. TOTAL RADIOACTIVITY OF SKIM MILK POWDER (cpm/g of Ash)

Locality		Number	1956 June	July	August	September	October	November	December	1957 January	February	April	May
Hokkaido		A 1		46	39	53	45	51	46			50	36
〃		B 2	44	45	38	41	47	40		26	33		
〃		C 3	46				45	47					
〃		D 4	49	49	49	50	45	40	31	41	42		46
Tôhoku	Iwate	5	47	41	41	41	41	46				48	
〃	Yamagata	A 6	40	46	44	43	44		50			42	38
〃		B 7	53	58	44	54	55	40			46	43	45
Kantô	Kanagawa	8	50	50	43	47	50	42	43		40		49
Chûbu	Nagano	A 9	41		38	51	38	49	45	40	40		
〃	〃	B 10	43		48	49	41	50	44	42		46	48
〃	〃	C 11	49	59	38	54	49	47	48	39			40
〃	Yamanashi	12		47	48	47	50	45	44				
〃	Shizuoka	A 13	48	45						41	39	38	37
〃	〃	B 14					41	40				42	
Kinki	Hyôgo	A 15	49	50	45				40				
〃	〃	B 16	46			43	43	50	48	44			44
Chûgoku	Okayama	17	45	46			43	50	47	43			43

counting was one hour both in background and in samples. The results are shown in Tables I and II.

II. Measurement of Sr-90 concentration in skim milk powder and sweetened condensed milk

(1) Samples

Skim milk powder (or in certain cases whole milk powder) collected from six districts every season (August, October in 1957, February, May in 1958) was used for the measurement of Sr-90 concentration. Besides these, sweetened condensed milk and skim milk powder manufactured before 1957 and 1956 respectively were also used for assay. But in the case of the latter, four kinds of skim milk powder collected from four districts were mixed in proportion to the output of each district, and then this mixture was submitted to assay.

(2) Separation procedure of Sr-90 and the measurement of radioactivity

(a) Outline of separation procedure: In the same way as discribed in Experimental I, the samples were burnt to ashes below 650°C.

100 g of ash were generally used for this assay, but in the case of the samples which contained Sr-90 in comparative large quantity, 20~50 g were used.

As to separation procedure, the nitric acid method, having been originated in Noll's method[1] and improved by Asari[2], was adopted.

Outline of this method is as follows. Carrier-free sample was dissolved in dilute fumic nitric acid. This solution was boiled and filtered. The filtrate was evaporated in order to remove chlorine gas. After cooling, fumic nitric acid ($d=1.52$) was added, and this solution was allowed to stand overnight, and filtered through sintered glass funnel. The precipitate obtained was dissolved in hot water and filtered. After the filtrate was evaporated, fumic nitric acid ($d=1.52$) was added. The solution was filtered as before. This procedure was repeated two or three times.

The precipitate was dissolved in hot water and evaporated below 100 ml. 1.5 M sodium chromate and ammonia were added to this solution. Then the solution was adjusted pH 5.5~6.0 with acetic acid, heated at 90°C, and filtered. After the filtrate was evaporated below 100 ml, ammonium acetate and barium carrier (barium chloride) were added. The solution was heated at 90°C, and filtered as before. The procedure was repeated once again. By this procedure barium and radium were separated from calcium and strontium.

Ammonia and excess of ammonium carbonate were added to the filtrate. The solution was boiled and filtered. The precipitate obtained was dissolved in dilute nitric acid and evaporated to dry up. The residue was dissolved in small amount of water and to this solution fumic nitric acid was added until the specific gravity became just 1.45. By this procedure only strontium nitrate was precipitated. The precipitate was dried and

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[2] T. Asari and H. Watanabe, *Reports of the Physical and Chemical Research Institute*, **23**, 402 (1944).

TABLE III. Sr-90 CONCENTRATION OF SKIM MILK POWDER OR WHOLE MILK POWDER

Kind of Sample	Locality		Date		Ca% in ash	Sr/100 Ca	Sr^{90}μμc/g of Ca (S.U.)	Sr^{90}μμc/7 mg Sr (M.U.)	Sr^{90}μμc/kg of Sample
Skim	Hokkaido		August	1957	14.4	0.038	4.3±0.4	79.4	50.1
〃	〃		October	〃	14.6	0.033	5.2±0.5	110.0	63.8
〃	〃		January	1958	14.7	0.062	6.7±1.5	76.5	82.2
〃	〃		May	〃	14.7	0.032	4.2±0.97	86.9	48.1
Whole	Tôhoku	Miyagi	August	1957	15.0	0.030	1.2±0.1	28.0	10.5
Skim	〃	Yamagata	October	〃	13.5	0.036	2.0±0.2	39.4	21.7
Whole	〃	〃	January	1958	15.4	0.023	0.9±0.06	28.5	8.8
〃	〃	〃	May	〃	13.2	0.027	1.7±0.85	44.2	13.8
〃	Kanto	Gunma	October	1957	15.1	0.021	1.1±0.2	35.5	10.1
Skim	〃	〃	January	1958	14.5	0.041	1.4±0.3	24.0	17.1
〃	〃	〃	May	〃	14.8	0.024	1.0±0.2	29.8	12.4
Whole	Chûbu	Yamanashi	August	1957	15.7	0.031	1.5±0.2	34.8	13.9
〃	〃	Nagano	October	〃	14.9	0.034	1.2±0.1	25.4	11.1
〃	〃	〃	January	1958	14.4	0.046	2.2±0.3	33.0	19.6
〃	〃	〃	May	〃	14.6	0.033	0.8±0.3	15.8	6.7
〃	Kinki	Hyôgo	August	1957	14.0	0.026	1.3±0.3	35.9	11.6
Skim	〃	〃	October	〃	14.5	0.025	0.61±0.12	17.3	7.6
〃	〃	〃	January	1958	14.5	0.041	1.0±0.25	17.4	12.5
〃	〃	〃	May	〃	15.1	0.019	0.5±0.1	16.4	5.8
〃	Chûgoku and Shikoku, Tokushima		August	1957	13.6	0.022	1.2±0.2	36.2	13.9
〃	〃	〃	October	〃	14.3	0.041	1.4±0.3	23.2	16.6
〃	〃	〃	January	1958	14.9	0.024	1.6±0.25	45.4	19.6
〃	〃	〃	May	〃	13.5	0.031	1.0±0.5	22.3	11.2

TABLE IV. Sr-90 CONCENTRATION OF SWEETENED CONDENSED MILK

Whole of Sample	Locality		Date		Ca% in ash	Sr/100 ca	Sr^{90}μμc/g of Ca (S.U.)	Sr^{90}μμc/7 mg Sr (M.U.)	Sr^{90}μμc/kg of Sample
Whole	Kanto	Chiba		1950	12.5	0.017	0.1±0.06	4.0	0.24
〃	Hokkaido		July	1956	14.4	0.05	2	38.0	6.7
Skim	〃		September	1957	14.8	0.043	3.4±1.0	54.4	12.2
〃	Kanto	Kanagawa	〃	〃	14.5	0.035	2.6±0.05	51.4	9.0

TABLE V. Sr-90 CONCENTRATICN OF SKIM MILK POWDER

Kind of Sample	Locality	Date	Ca% in ash	Sr/100 Ca	Sr^{90}μμc/g of Ca (S.U.)	Sr^{90}μμc/7 mg Sr (M.U.)	Sr^{90}μμc/kg of Sample
Skim	Hokkaido Yamagata Ishikawa Hyôgo	1951 1952 1953	14.0	0.018	0.054±0.03	2.1	0.65
Skim	Hokkaido Yamagata Ishikawa Hyôgo	1956	14.4	0.07	2.1	21.0	2.2

weighed. After weighing, the strontium nitrate was converted to carbonate, and then submitted to counting.

This strontium carbonate was left for two or three weeks until radiation equilibrium was obtained between Sr-90 and Y-90, and then dissolved in hydrochloric acid. To this solution ferric chloride was added as carrier for measurement of yttrium, and carbonic acid free water saturated with ammonia was added. Yttrium was precipitated as yttrium hydroxide and this precipitate was submitted to counting.

(b) Measurement of radioactivity : Radioactivity was measured at the Yamazaki Laboratory of the Physical and Chemical Research Institute. The results are shown in Tables III, IV and V.

Sr-90 concentration in samples is given as Sr-90$\mu\mu$c/g of Ca (S. U., Sunshine Unit). And, in addition, Sr-90 $\mu\mu$c/kg of sample was calculated for convenience.

DISCUSSION

I. Total radioactivity

It was found that there was scarcely any difference in the total radioactivity between raw milk and skim milk powder. The values shown in Tables I and II were averaged monthly as shown in Table VI.

TABLE VI. TOTAL RADIOACTIVITY AVERAGED MONTHLY

Date Kind of Sample	1956 June	July	August	September	October	November	December	1957 January	February	April	May
Raw milk	48	50	44	45	47	46	47	42	45	—	44
Skim milk powder	46	47	43	48	45	46	45	40	40	44	43

Table VI shows that seasonal variation in total radioactivity is hardly found in both raw milk and skim milk powder. And also regional variation is hardly noticeable in Table I and II.

From these results, it is reasonable to expect that most of the radioactivity in milk could be attributed to natural radioactive substances and therefore variation in radioactivity owing to artificial radioactive substances, if any, hardly affects the total radioactivity.

In order to ascertain this, simple experiment was carried out. 0.5 g of potassium chloride, which was commonly used as reagent in chemical analysis, was weighed out into counting tray and its radioactivity was measured in the same way as mentioned in the measurement of radioactivity.

As a result, the radioactivity of potassium chloride showed 118 cpm per 1 g. If all of the radioactivity of potassium chloride is originated in potassium, the radioactivity of potassium will be 226 cpm per 1 g. As to potassium sulfate, the same result was obtained. Further, it was shown by Nakae[3] that the content of potassium in milk ash was averaged 0.21 g per 1g of ash. From these results, consequently, it is recongnizable that the radioactivity of potassium in 1 g of milk ash become about 48 cpm.

Bergh[4] showed that most biological materials contained appreciable K-40 activity, in case of milk 3000～5000 dpm per liter. In authors' experiment, 40～50 cpm per 1g of ash is converted to 2400～4000 dpm per 1 liter of milk.

Putting all these results together, it is considered that most of the radioactivity in milk would originate in natural radioactive substances, especially such as K-40.

It is considered that this is the reason why neither seasonal variation nor regional variation in total activity was found in this experiment.

II. Sr -90 concentation

Table III shows that Sr-90 concentration in skim milk powder is higher in the northern districts of Japan than in the central or southern districts and the highest in Hokkaido.

A report from the Japan Meteorological Agency[5] showed that the accumulative amount of Sr-90 (presumtive value) was higher in Hokkaido and Tohoku (the north-east district of Honshu) than on the Pacific side of Japan. It is considered that this is one of the reasons why Sr-90 concentration in skim milk powder is higher in Hokkaido or in the north-east. Besides, the condition of feeding cows is considered as another reason.

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[3] T. Nakae, *Bulletin of the National Institute of Agricultural Sciences, Japan*, No. 36 (1938).

[4] H. Bergh, A Method for the Determination of Very Small Amounts of I-131 in Biological Materials, Expecially in Milk A/CONF. 15/P/586, Norway, 28 May, 1958.

[5] The present condition of observation of artificial radioactivity in Japan, March 1958: Reports of Japan Meteorological Agency.

In Hokkaido the grazing of cows is practised extensively, while in the other districts of Japan cows are usually fed in stalls. In case of grazing, there are more frequent occasions that cows directly take grass which is contaminated by radioactive rain water and directly exposed to radioactive rain water or dust than in case of feeding in a stall. Therefore it is considered that the milk produced in Hokkaido contains more Sr-90 than that in the other districts of Japan.

A report from the United National General Assembly[6] showed that the average Sr-90 concentration in milk produced in six stations of Canada was 5.0 S.U. in 1956 and 6.2 S.U. in 1957. These are similar values in Hokkaido, but are much higher than in the other districts of Japan. And the same report showed the average Sr-90 concentration in milk produced in New York City was 3.9 S.U. in 1956 (from January to May) and 4.5 S.U. in 195^7 (from January to August) and, further, average Sr-90 concentration in milk produced in Mandan was 9.2 S.U. in 1956 and 9.3 S.U. in 1957 (from January to July).

From above, it is recognized that Sr-90 concentration in milk is higher in Mandan than Hokkaido.

A report from West Germany[7] showed that the rate of radioactive contamination on milk produced in West Germany was similar to that in the United States of America and Canada, and that the averages Sr-90 concentration in milk was 4∼5 S.U. in 1956.

Putting these data together, it is conclusively evident that the milk produced in Japan is less contaminated by Sr-90 than in Canada, the United Siates of America and West Germany.

On the other hand, summarizing all results (shown in Tables III, IV and V) it is recognizable that the radioactive contamination on condensed milk and skim milk powder has shown an increasing tendency in its accumulation for the past decade in Japan. For instance, the Sr-90 concentration in condensed milk manufactured in 1956 is twenty times as high as that 1950.

But, in comparison with other foodstuffs, Radiological Data in Japan II[8] showed that the Sr-90 concentration in milk and milk products was much lower than in vegetables, tea, soybean and rice. Vegetables and cereals are directly contaminated by radioactive substances, while milk is filtered by passing through the cow's body. This is the reason why Sr-90 concentration in milk and milk products is lower than that in vegetables and cereals.

Table III shows that there is some seasonal variation in Sr-90 concentration, but judging merely from the results obtained, it is not possible to say about the relation between seasonal variation and atomic weapon test. As to this point, further investigations are required.

In order to account for the fact that Sr-90 concentration in skim milk powder manufactured in Hokkaido is higher than that in the other districts of Japan, it is necessary to investigate the Sr-90 concentration in food-stuffs, rain water and soil near the milk products factory in detail.

SUMMARY

Raw milk and skim milk powder collected monthly from several districts in Japan (from June 1956 to May 1957) were burnt to ashes and their total radioactivities were measured.

As a result, total radioactivities showed 40∼50 cpm per 1 g of ash. There was neither seasonal variation nor regional variation in the total radioactivity.

Skim milk powder (or whole milk powder) collected every season from six districts in Japan

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[6] United Nations General Assembly, Scientific committee on the effects of atomic radiation, The effects of atomic radiation, A/AC. 82/R. 65/Add. 3 8 May 1958.

[7] Physikalisches Institut der Bundesforschungsanstalt für Milchwirtsschaft, Kiel, Private communication (1958).

[8] Y. Hiyama, Radiological Data in Japan II. Concentrations of Sr-90, Cs-137, Pu-239 and others in various materials on earth's surface. Sept. 1958.

(August, October in 1957, Fubruary, May in 1958) was burnt to ashes and its Sr-90 concentration was measured.

Results obtained showed that Sr-90 concentration in skim milk powder manufactured in the northern districts were higher than those in the central or southern districts of Japan.

The Sr-90 concentrations are as indicated in the following districts.

Hokkaido : 4.2~6.7 Sr-90μμc/g of Ca
Tohoku : 0.9~2.0 〃
Kanto : 1.0~1.4 〃
Chubu : 0.8~2.2 〃
Kinki : 0.5~1.3 〃
Chugoku and Shikoku : 1.0~1.6 〃

The authors wish to thank Dr. Yamazaki and Mr. Hashizume, Physical and Chemical Research Institute, for having taken charge of counting in the measurement of Sr-90 concentration, and to Dr. Asari, Japan Inspection Co. Ltd., for his valuable counsel and good offices given in the measurement of Sr-90 concentration.

Crystallization of Taka-maltase

Sir:

Crystalline maltase has been isolated by the authors[1] from *Aspergillus oryzae* and by Tujisaka et al.[2] from *Aspergillus niger*. Some properties of this enzyme were reported, including a study on a transglucosidation action[2,3] and a comparative study of maltase activity with saccharogenic amylase[1,2].

In our method of crystallization from polished rice "Koji" the complete removal of the amorphous material was very difficult even after recrystallization. Taka-diastase "Sankyo" was used as source material and the authors have succeeded in the separation of maltase from other diastic enzymes and its crystallization by further purification (Fig. 1). The first few steps were essentially the same as those of Akabori et al.[4].

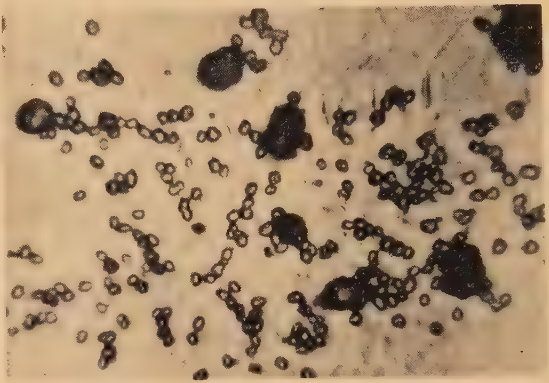

FIG. 1. Crystalline Takamaltase

Takadiastase (200 gms)

| Extracted with water and treated with 10% Ca-acetate (final conc. 1%), centrifuged (3,000 r.p.m., 10 min.)

Supernatant

| Added $(NH_4)_2SO_4$ (60 gms/100 ml), centrifuged (3,000 r.p.m., 30 min.)

Precipitate

| Dialyzed and treated with rivanol solution, centrifuged (2,000 r.p.m., 5 min.)

Supernatant

| Added $(NH_4)_2SO_4$ (60 gms/100 ml), filtered

Precipitate

| Dialyzed and diluted with equal volume of M/15 citrate buffer (pH=4.0)

Maltase and saccharogenic amylase fraction

| Chromatographed on Dowex-50-2 (Fig. 2)

Maltase fraction (No. 22 or No. 25)

| Dialyzed, precipitated with alcohol (75%), centrifuged (3,000 r. p. m., 10 min.)

Precipitate

| Dissolved in water, added alcohol dropwise to 60% concentration

Crystalline Maltase

Recrystallize from alcohol at 50% concentration

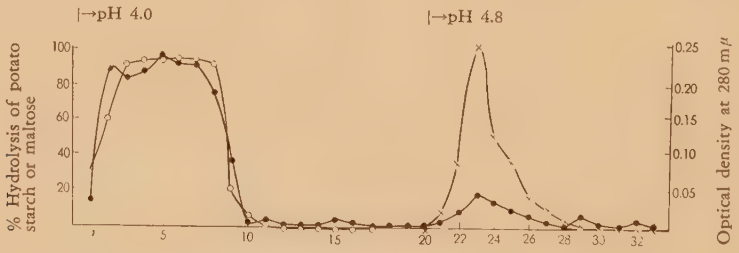

FIG. 2. Chromatography of Maltase-saccharogenic Amylase Mixture on a Column of Dowex 50-2.

●——● Optical density at 280 mμ with 0.1 ml of eluate.
○——○ Sacch. amylase activity with 0.5 ml of pH 4.0 eluate.
×——× Maltase activity with 0.2 ml of pH 4.8 eluate.

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[1] S. Sugawara, Y. Nakamura and T. Shimomura, This Bulletin, **23**, 156 (1959).
[2] Y. Tujisaka and J. Fukumoto, *Symp. on Enz. Chem. Japan* **10**, 84 (1958).
[3] S. Sugawara, Y. Nakamura and T. Shimomura, in press (in this Bulletin).
[4] S. Akabori, T. Ikenaka and B. Hagihara, *J. Biochem*, **41**, 583 (1954).

The specific activities and the yields in each step were as shown in Table I. Substrate specificity and transglucosidation action of crystalline Taka-maltase were identical with those of the crystalline enzyme isolated from other sources. However, the optimun pH was 4.5 which is quite different from optimun pH 3.5 for crystalline maltase of *Aspergillus oryzae* isolated by the authors.

The authors are indibted to Sankyo Co Ltd., for kindly supplying Takadiastase used as source material.

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TABLE I. PURIFICATION OF TAKAMALTASE

	Volume	U. N./0.5 ml	U. N./mgN	Yield
Original extract	2000 ml	1.2	——	100 %
1st. $(NH_4)_2SO_4$ ppt	1500	1.0	0.01	60∼70
After rivanol treatment	1700	0.9	0.04	55∼60
2nd. $(NH_4)_2SO_4$ ppt	150	9.8	——	50∼60
Dowex 50 treatment (No. 22∼25)	80	7.8	34	30∼35
Alcohol ppt (75 %)	15	22.0	35	20∼30
1st. crystallization	10	16.7	40	10∼25

1 U. N.: mg glucose liberated from phenol-α-glucoside/0.5 ml enz. sol/hr.

[Bull. Agr. Chem. Soc. Japan, Vol. 24, No. 1, p. 113∼114, 1960]

Dietary Protein and Cholesterol Metabolism

The Effect of Amino Acids Supplementation on Serum Cholesterol Level

Sir:

Recently much attention has been focussed on the effects of dietary protein on serum cholesterol level[1~6]. A majority of these studies were carried out under the experimental condition of administering cholesterol. Our study included primarily the effect of dietary protein of different source and quantity on serum cholesterol concentrations in the rats, especially under the feeding condition, where no cholesterol was given. The results showed that the difference in the serum cholesterol level was the greatest between rats fed with 5 %- and 30 %-casein diet, the serum cholesterol concentration being 138 mg% and 76 mg%, respectively. The difference in the serum cholesterol level between rats fed with 5 %- and 30 %- soy protein diet was the next, serum cholesterol being 113 mg% and 83 mg%, respectively. The serum cholesterol decreased in those animals fed with high protein diet. But when fed with 5%- and 30 %-gluten diet, the serum cholesterol level showed no change in spite of different protein content, cholesterol level being 104 mg% and 99 mg% respectively. These results indicate that serum cholesterol level is influenced by the quality of protein.

Then we made comparison of amino acids composition between casein, soy protein and gluten, and the effects of amino acids supple-

TABLE SERUM CHOLESTEROL LEVELS ON RATS FED THE DIETS SUPPLEMENTED WITH AMINO ACIDS

Group	Basal Diet[i]	No. of Rats	Serum Ch on 10th day after feeding Basal Diet (mg%)	Supplemented Amino Acids	Serum Ch on 34th day after Amino Acids Supplementation (mg%)
I	5% Gluten	3	105±3[ii]	0.4% L-Lysine	93±13
II	30% Gluten	5	103±7	2.0% L-Lysine	70± 4*
III	5% Gluten	4	98±5	0.4% L-Lysine 0.2% DL-Threonine	109± 1
IV	30% Gluten	5	97±4	2.0% L-Lysine 1.3% DL-Threonine	70± 3**
V	5% Casein	5	104±1	0.5% Glycine	121± 3
VI	30% Casein	5	65±3**	3.0% Glycine	58± 3**

Ch: Cholesterol ** Significance $P<0.1$ * Significance $P<5$

i) Basal Diet: McCollum Salts 4g, Sesame Oil 5g, Vitamin Mixture (Thiamine 1.0mg, Riboflavin 1.0, Ca-Pantothenate 3, Pyridoxine 2, Niacin 2.5, Folic acid 0.2, Biotin 0.04, Choline 100, Inositol 10, Vitamin A 5000 I.U., D 1000 I.U. per 100g Diet) various Proteins made up to 100g with Starch

ii) Standard Error of the Mean

mentation on serum cholesterol level was examined. Lysine, which is contained in gluten in lower percentage than in casein and soy protein, was supplemented to the gluten diets so as to make the same content as in 5%- and 30%-casein diet. Referring to the report of Sure[7], threonine was added in addition. Furthermore in order to study the effects of glycine contained in gluten a little more than in other protein, experimental group V and VI were included. Male rats of the Wistar strain, weighing about 150 g, were used. Animals were fed with basal experimental diet without supplementation of amino acids for first 10 days and serum cholesterol determination was carried out on the 10th day. As shown in the Table, the serum cholesterol in animals fed with high casein diet decreased, a result which is the same as obtained in the preceding experiment just mentioned. The second serum cholesterol determinations were made on the 34th day after amino acids supplementation. The Table shows that significant difference in serum cholesterol level was caused between Group I and II, III and IV and V and VI. It was found that serum cholesterol level showed marked difference between low and high gluten diet, a finding which has never been observed previously. Reduction of serum cholesterol in Group II and IV was not related to threonine but it was caused by lysine supplementation. Effects of glycine was not particularly significant and it was thought that the response by gluten, which was considered to be peculiar, was not due to glycine in excess.

Elvehjem et al.[1], reported that 1% lysine supplementation to 30% gluten diet had no effect on serum cholesterol level under cholesterol administration, but study showed that 2% lysine supplementation equalled to 30% casein value caused significant reduction of serum cholesterol level. Thus amino acid balance is closely related to cholesterol metabolism and it seems to be very interesting problem to investigate further.

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Synopses of the Articles printed in NIPPON NÔGEI-KAGAKU KAISHI
(In Japanese)
Vol. 33, No. 12 (1959)

Non-microbial Deterioration of Preserved Egg Albumen during the Storage. Part IV. Dialyzable Sugars of Preserved Egg. (p. *997～1000*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

The studies to be reported herein were conducted to provide some information relative to the process of denaturation or decomposition of mucoproteins of chicken egg during the storage. Albumen and yolk dissolved in 10% NaCl solution, prepared from fresh egg and eggs preserved at 38°±1° and 0°～5° for 21～28 days, were dialyzed against distilled water at 0°～5° for 5～10 days. The sugars in dialysates were detected by paper chromatography. D-Glucose, D-mannose, D-fructose, D-xylose, two unidentified sugars and a sugar whose R_{glucose} value was as same as that of potassium salt of D-glucose-6-sulfate ester were detected in dialysates of both fresh albumen and yolk and also L-arabinose, an unidetified polysacaccharide were detected in dialysate of albumen and two unidentified polysaccharides, one of which was aminosugar, were detected in that of yolk. Some of these dialysable sugars might exist as free and some of them might be weakly bound in fresh albumen and yolk. It was concluded that these bound sugars were liberated gradually during the storage and the bound sugars of albumen were more strongly bound than these of yolk. The liberation of these sugars and the difference of their bonding forces between these of albumen and yolk were also observed during the decomposition of these materials by 0.1 N NaOH at 40°.

Sulfate-sulfur in Chicken Egg. Part VI. Glycosulfatase in Embryonated Egg. (p. *1000～1005*)

By Yoshinobu NONAMI

(*Faculty of Agriculture, University of Niigata*)

This study was undertaken in order to throw further light on the role of free and weakly bound sulfate-sulfur in allantoic and amnionic fluids of embryonated egg, and to get some informations concerned with fate of all sulfate-sulfur in yolk and white during incubation. Condrosulfatase and glycosulfatase in allantoic, amnionic fluids and all other differentiated parts of embryonated eggs were determined at the period of 120, 210, 330, 400 hours of incubation. Condrosulfatase was never identified quantitativly in any parts of the eggs, but considerable amount of glycosulfatase was detected in allantoic and amnionic fluids. It was observed that the unidentified sugar whose R_{glucose} value on paper chromatograms was as same as that of potassium salt of D-glucose-6-sulfate ester, was hydrolysed by 0.1 N HCl and also by glycosulfatase of allantoic or amnionic fluids prepared from embryonated eggs incubated for 330 hours, giving D-glucose and sulfate ion as products of the hydrolysis. The paper chromatograms of dialyzable sugars of yolk and white prepared from embryonated eggs, indicated that the glucose-sulfate compound disappeared from the dialyzates of white at the period of 330 hours of incubation, when all sulfate-sulfur might change significantly in embryonated egg and that at the period of 210 and 330 hours of incubation, none or very little amount of dialyzable sugars existed in both yolk and white, but at 400 hours of incubation, there were many kinds of dialyzable sugars in yolk. It was concluded that most significant change of sugars and sulfate-sulfur occured after 330 hours of incubation in embryonated egg especially in the tissues surrounding the embryo.

Studies on the Fermented Milk. Part IV. Studies on Stabilizers of the Sour Milk Drink containing Natural Fruit Juice. (IV) On the Mechanism of the Coagulation and on the Stabilizing Effect of Various Stabilizers. (p. *1005～1010*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co., Ltd.*)

When the sour milk drink is mixed with natural fruit juice, the milk protein and the suspending ingredient of juice coagulate instantly. The mechanism of this coagulation is investigated. The mobilities of milk protein and juice ingredient are examined by means of the electrophoresis. Simultaneously the mobilities and hydrations of following stabilizing agents, Propylen glycol alginate, Sodium alginate, Locust bean gum, Gum arabic, Gum tragacanth, Agar-agar, Pectin, Methyl cellulose, Sodium carboxymethylcellulose, Polyvinylpyrrolidone are examined. With above stabilizers, the sour milk drink containing natural fruit juice is prepared and these drinks are stored for the examination of the stability. These results are taken as an indication that:

(1) Without the stabilizer, positive particles in sour milk and negative particles in juice combine with each other.
(2) It seems that the stabilizer covers over these combined particles and forms the protective colloid and stable suspension.
(3) Excellent properties as a stabilizer of such drink that contains milk protein and natural juice show as follow :
 a) Execellent stabilizers have higher Newtonian viscosity and higher hydration.
 b) Stabilizer's solution is stable at pH 3.0∼4.0.
 c) The mobility in electrophoresis at pH 3.2 is relatively low so far as these aboue stabillizers concerned.

Microdiffusion Analysis for Determination of Alcohol in Shoyu. (Application of Microdiffusion Analysis to Brewing. II.) (p. *1010∼1013*)

By Yuzuru KUME, Yoichi IMURA and Kimiyoshi SEKIYA

(*Fundokin Shoyu Co., Ltd.*)

The Conway's method for determining alcohol in blood or other fluid is modified to apply to the analysis of shoyu.

A thorough statistical study was made on removing assignable causes, on precision and accuracy, on comparison of results with the usual methods, and on the elimination of interfering substances. A coefficient of variation of the method is ±0.520% at the 30∼289 mg % concentration level.

Biochemical Studies on the Rice Blast Disease. Part X. The Biochemical Classification on *Piricularia oryzae* CAVARA. (7) On the Production of Vitamin B Group by *Piricularia oryzae* CAVARA. (p. *1013∼1018*)

By Hitoshi OTSUKA

(*Faculty of Education, University of Niigata*)

The strains of *Piricularia oryzae* CAVARA which were able to grow on thiamine free synthetic media cultured on thiamine free synthetic media, little thiamine contents in its mycelium and culture broth were formed.

The strains of *P. oryzae* which were able to grow on synthetic media when nicotinic acid was added instead of thiamine cultured on synthetic media to which nicotinic acid was added instead of thiamine, little thiamine contents in its mycelium and culture broth were formed too. All the strains of *P. oryzae* secreted the folic acids riboflavin, pantothenic acid, vitamin B_6 group in culture broth when cultured on perfect synthetic media. The effect of botin on nicotinic acid secretion through *P. oryzae* was investigated as well.

Studies on the Carbohydrates of Allium Species. Part IV. Free Sugars and Polysaccharides of *Allium cepa* Linn. (p. *1018∼1025*)

By Takashi MIZUNO, Teruhisa KITANISHI and Tadao KINPYÔ

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University, Iwata*)

Carbohydrates of four portions of onion, *Allium cepa* Linn., i.e., green leaf, middle leaf, bulb and root, were divided respectively into seven fractions by the extraction in the following order : hot 80% ethanol (free sugars fraction), hot 50% ethanol (I), cold water (II), hot water (III), hot 0.5% ammonium oxalate solution (IV), cold and hot 5% sodium hydroxide solution (V and VI). Free sugars and the component sugars of each fraction were investigated mainly by the densitometric method of paperchromatogram. The following results were obtained.

Considerable amounts of free sugars were found in each portion, except in root, and their total contents in anhydrous matter were 35% in green leaf, 56% in middle leaf, 68% in bulb, trace in root. Glucose, fructose, saccharose and from two to five kinds of fructoöligosaccharide composed only of fructose unit, were detected in each portion from free sugars fraction. In addition, lower molecular fructan and glucofructan were detected in bulb portion.

Respective polysaccharides were isolated from above six fractions (I∼VI); details and molecular ratios of their component sugars were made clear as follows.

In green leaf portion, polysaccharide I composed of galacturonic acid (Gau) : rhamnose (Rh) : arabinose (Ar) : galactose (Ga) = 1 : 2 : 4 : 16, II was Gau : Rh : Ar : Ga = 1 : 5 : 6 : 11, III was Gau : ribose (Ri) : desoxyribose (Deri) : Rh : Ar : Ga = 1 : 5 : 5 : 11 : 39 : 46, and IV was Ar : Gau : Ga = 1 : 31 : 93.

In middle leaf portion, polysaccharide I composed of Rh : Ri : Ar : Gau : Ga = 1 : 1 : 2 : 2 : 9, II was Gau : Ar : Rh : Ga = 1 : 8 : 10 : 17, and Ga, Ar, Gau and Ri in III, Ga, Rh, Ar, Gau in IV were detected as the component sugars respectively.

In bulb portion, Ar : Gau : Ga = 1 : 1 : 11 in I, Gau : Rh : Ar : Ga = 1 : 5 : 11 : 24 in II, Gau : Ar : Rh : Ga = 1 : 10 : 10 : 23 in III, and Ga, Gau, Ar, Rh in IV were detected respectively.

In root portion, I composed of Gau : Deri : Rh : Ri : Ar : Ga = 1 : 2 : 6 : 11 : 13 : 26, II was Ri : Rh : Ar : Ga = 1 :

5 : 10 : 13, III was Deri : Ri : Gau : Rh : Ar : Ga = 1 : 4 : 5 : 6 : 12 : 21, and Gau, Ga, Ar, Rh in IV were detected.

Ga only was detected as the component sugar of polysaccharides V and VI isolated from any portion.

Studies on the Components of *Ficus carica* L. Part II. Furocoumarins from the Root.

(p. *1025～1027*)

By Shun-ichi FUKUSHI

(*Department of Agriculture, University of Tottori*)

The isolation of three crystalline substance, $C_{12}H_8O_3$ (I) m.p. 160～161°, $C_{13}H_{10}O_4$ (II) m.p. 182～183°, $C_{13}H_{11}O_3$ (III) m.p. 140～142°, from the volatile oil of the root of *F. carica* L. on a alumina column is described.

On the basis of ultraviolet absorption spectra in ethyl alcohol and chemical properties, they appear to be furocoumarins.

The spectrum of I is closely similar to that of psoralene and the spectrum of II is the same type of bergaptene.

Studies on the Components of *Ficus carica* L. Part III. On the Essential Oil of the Green Leaves.

(p. *1028～1030*)

By Shun-ichi FUKUSHI

(*Department of Agriculture, University of Tottori*)

As the volatile components of the green leaves of *Ficus carica* L., palmitic acid, valeric (or isovaleric) acid, guaiacol, octacosane, tricosane (or tetracosane), *p*-cymene, a kind of ester, and sesquiterpene, were newly isolated.

When the sesquiterpene was dehydrogenated with powdered S, a few amount of cadalene was obtained.

Researches on Ergot Fungus. Part XXIX. Isolation of Two New Water-Soluble Ergot Alkaloids, Secaclavine (Alkaloid „X″) and Festuclavine (Alkaloid „Y″), from the Sclerotia and Saprophytic Cultures of Ergot Fungi.*

(p. *1031～1036*)

By Matazō ABE and Saburō YAMATODANI

(*The Institute for Fermentation, Osaka, Japan*)

The ergot alkaloids „X″ and „Y″ formerly detected by paper partition chromatography were isolated for the first time in crystalline form, utilizing the differences of their distribution coeffecients between two solvents immissible with each other. „X″, $C_{16}H_{20}ON_2$, m.p. 218～220° (uncorr. decomp.), $[\alpha]_D = -232°$ (in pyridine), was isolated from the ergots of the *Elymus*, *Phragmites*, *Phalaris* and *Agropyrum* growing in Japan and the Spanish ergot of rye as well as from the saprophytic cultures of the ergot fungi obtained from those ergots, while „Y″, $C_{16}H_{20}N_2$, m.p. 239～240° (uncorr. decomp.), $[\alpha]_D = -107°$ (in pyridine), was isolated from the cultures of the fungi obtained from the ergots of the said *Phaloris* and *Agropyrum* and from the ergot itself of the latter. „X″ and „Y″ were proved to be new water-soluble alkaloids, for which the authors proposed the names, secaclavine and festuclavine, respectively. Secaclavine was presumed to be a sort of dihydrolysergol, while festuclavine was identical in all respects with the dihydro-derivative obtained in the previous work by catalytic hydrogenation of agroclavine.

* Part of this paper was preliminarily reported in *J. Agr. Chem. Soc. Japan*, **28**, 501 (1954).

Researches on Ergot Fungus. Part XXX. Chemical Relationship between Elymoclavine and the Hitherto Known Peptide-Type Ergot Alkaloids.* (p. *1036～1039*)

By Saburō YAMATODANI and Matazō ABE

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Previously, Abe et al.[1] reported that their elymoclavine yielded on catalytic hydrogenation a dihydro-derivative, which had a close resemblance to α-dihydrolysergol. The present authors have succeeded in obtaining from the mother liquor of this dihydro-derivative an additional dihydro-derivative in very small yield, and, at the same time, have demonstrated that the previously reported dihydro-derivative and the newly obtained one were just identical with α-dihydrolysergol and β-dihydrolysergol, respectively, in crystal form, melting point, specific rotation, paper chromatographic behavior and IR.-spectrum. From these results, the authors have not only evidenced the reasonableness of the structure of elymoclavine, previously presumed by Abe et al.[1], but also clarified the chemical relation of elymoclavine to the so-called peptide-type ergot alkaloids.

* This paper was preliminarily reported in This Bulletin, **19**, 94 (1955).

[1] M. Abe, T. Yamano, Y. Kôzu and M. Kusumoto, *J. Agr. Chem. Soc. Japan*, **25**, 458 (1952); **29**, 364 (1955).

Researches on Ergot Fungus. Part XXXI. Isolation of Penniclavine and a New Water-Soluble Ergot Alkaloid, Triseclavine, from the Saprophytic Culture of the Elymus-Type Ergot Fungus.* (p. *1039～1043*)

By Matazō ABE, Saburō YAMATODANI, Tōgo YAMANO and Mitsugi KUSUMOTO

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Two unknown ergot alkaloids „A″ and „B″ were isolated from the saprophytic culture of the *Elymus*-type ergot fungus parasitic on Japanese *Elymus mollis* Trin., applying starch-column partition chromatography. „B″ was isolated in excellent yield, also, from the saprophytic culture of the *Agropyrum*-type ergot fungus parasitic on the *Agropyrum*, *Trisetum*, *Festuca* etc. growing in Japan.

Both „A″ and „B″ gave a blue color with concd. sulfuric acid, but a green color with van Urk's and Keller's reagents. „A″, $C_{16}H_{18}O_2N_2$, m.p. 219°C (uncorr. decomp.), $[\alpha]_D = +154°$ (in ethanol), $[\alpha]_D = +150°$ (in pyridine), was identical in all respects with penniclavine reported by A. Stoll et al., while „B″, $C_{16}H_{18}ON_2$, m.p. 228～230°C (uncorr. decomp.), $[\alpha]_D^{20} = +165°$ (c. 0.2 in ethanol), $[\alpha]_D^{20} = +172°$ (c. 0.2 in pyridine), was proved to be a new water-soluble alkaloid, for which the authors have proposed the name triseclavine. It was presumed that penniclavine and triseclavine might be produced by the fungus through elymoclavine and agroclavine, respectively.

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* This paper was preliminarily reported in This Bulletin, **19**, 92 (1955).

Studies on the Bitter Taste Peptides produced by Proteinases. Part I. The Formation of Bitter Taste Peptides by "Neutral Proteinase" of *Bacillus subtilis* and Isolation of the Peptides. (p. *1044～1048*)

By Kazuhiro ICHIKAWA, Takehiko YAMAMOTO and Juichiro FUKUMOTO

(*Institute of Polytechnic, Osaka City University*)

Protein digestion by bacterial proteinases was found to proceed forming bitter taste peptides and hence the condition of formation of the bitter taste peptides and the method of isolation of the peptides were investigated. In the experiment, "Neutral proteinase" of *Bac. subtilis* was used as the proteinase. The proteinase produced the peptides most abundantly at casein digestion. The amount of the peptide formed was found not always to go parallel with the degree of protein digestion. The formation of the peptides was most remarkable in the vicinity at pH 6.0. The peptides were isolated in the following method: the casein digests by the proteinase was treated with barium chloride and alcohol successively, in order to remove phosphopeptone following the Peterson-Nauman-McMeekin's method (*J. Am. Chem. Soc.*, 80, 95 (1958)). The filtrate thus obtained was concentrated and fractionated by the counter current distribution method using butanol/water as the solvent. The both preparations obtained from the butanol and aqueous layer gave bitterness respectively. The bitter taste peptide found in the butanol layer was found to be dialysable and was isolated as an electrophoretically pure peptide. The bitterness of this peptide was not lost by heating, but was easily lost when heated at a slight acid or alkaline condition.

Study on the Nitrate Reductase. Part XVIII. Decrease of Nitrite by Cattle Spleen. (p. *1048～1050*)

By Hirohisa OMURA, Sadao MIZOGUCHI and Haruki SHIGEMATSU

(*Institute of Agricultural Chemistry, Kyushu University*)

Decrease of nitrite by the homogenate of cattle spleen was observed. The activity was precipitated between 33% and 55% of acetone, but not between 55% and 70%. On the other hand, the activity was established in the enzyme preparation which was deposited between 0.2 and 0.5 saturation of ammonium sulfate. The decrease of nitrite as well as the reduction of nitrate by this fraction was accelerated by acetaldehyde.

Studies on the Soluble Wool Protein. Part I. Electrophoresis, Sedimentation and Light Scattering of α-Keratose. (p. *1051～1054*)

By Yoshimitsu MIYAZAKI

(*Faculty of Agriculture, Saga University*)

(1) At higher protein concentration (above 0.66%) of α-keratose in carbonate buffer, the asymmetric pattern could be observed on the descending boundary and disappeared at low protein concentration (0.39%). On the other hand the pattern of α-keratose in veronal was symmetry even at the higher protein concentration (1.6%).

These facts and the measurements of light scattering which showed a little variation in veronal and greater in carbonate against the change of protein concentration suggested that there may be a difference in the modes of agregation according to the kind of the buffer employed.

(2) The sedimentationp attern in veronal buffer (pH 8.6, μ0.1) showed a single peak, and this fact showed that α-keratose was a homogeneous component. The value of sedimentation coefficent $s_{20w} = 2.5S$ was obtained.

(3) From the measurements of light scattering of α-keratose the curve of Hc/τ virsus protein concentration was obtained and it was extrapolated to infinite dilution. The particle weight was accounted for 500,000 in both veronal (pH 8.6, μ0.1) and carbonate (pH 10.7, μ0.1) buffers. When the 5 M urea was added in these buffers, particle weight appeared to be 105,000 in veronal, but the extrapolation was not successful in carbonate buffer.

Studies on Honey. Part II. Isolation of Kojibiose, Nigerose, Maltose and Isomaltose. (p. *1054～1058*)

By Toshiyuki WATANABE and Kiyoshi ASO

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Honey (*Lespedeza bicolor*) was fractionated by a carbon-Celite column using water and 2.5～30% ethanol as successive elution solvents. The effluent portions containing kojibiose and isomaltose (2.5～5.0% ethanol fraction) were then rechromatographed on a carbon-Celite column using the gradient elution method with 0～3.0% aqueous ethanol containing borate buffer (pH 10.0). Kojibiose fraction (2.0～3.0% ethanol) and isomaltose fraction (0.5～1.0% ethanol) were acetylated as usual and then the two sugars were isolated as their crystalline octaacetates by Magnesol-Celite column chromatography. Nigerose and maltose were also isolated as their crystalline octaacetates by a carbon-Celite column and Magnesol-Celite column chromatographic procedures.

Studies on the Microbiological Assay of B-vitamines and Amino Acids by the Pulp Disc Methods. Part VI. On the Assay of L-Threonine by *Streptococcus faecalis R.* (p. *1059～1062*)

By Masaya HAYASHIBE, Takao SUGIHASHI, Takamichi SAEKI and Akiyoshi ICHIBA

(*Osaka Laboratory, Nisshin Flour Milling Co.*)

Yoshitsugu FUJII and Teijiro UEMURA

(*Department of Agriculture, Tohoku University*)

The microbiological Assay of L-threonine by the pulp disc method was previously reported using *Leuc. mesenteroides* as a test organism. This organism when employed does not give any clear growth zone, and thus is not suitable to this assay method. Then, *Str. faecalis R* was chosen as a test organism which offered much clearer growth zone in a preliminary experiment. The assay conditions of L-threonine by *Str. faecalis R* were examined and settled. The conditions of a rapid determination by which L-threonine was determined after 4 to 5 hours' incubation, was also settled. When L-threonine in the fermented broth with *B. subtilis* strain No. 14 was assayed by this method, a nomal growth zone did not appear owing to the antibiotics by-produced by this strain, that is, growth inhibiting zone appeared around the pulp disc while growth zone outside of it, resulting in much higher value of L-threonine. In order to remove such concomitant antibiotics, the cultured filtrate was treated with active charcoal, and thus good results were obtained.

Studies on Flavonoid. Part IV. The Position of Hydroxy Group chelating with Aluminium Chloride. (p. *1063～1065*)

By Keizo HAYASHIYA

(*School of Textile Fibers, Kyoto Technical University*)

The ultra-violet absorption maxima of flavonols undergo major shifts towards longer wavelengths by the addition of aluminium chloride to these alcoholic solutions. The 3-hydroxy group in flavonols is essential for these effecting changes in the absorption spectra. The 5-hydroxy group in flavonols, being involved in chelation with 4-keto group, is not that depending on the changes which take place in their spectra on addition of alcoholic aluminium chloride to their alcoholic solutions.

Studies on Flavonoid. Part V. The Synthesis of 7,5,4'-Trimethoxymorin. (p. *1065～1068*)

By Keizo HAYASHIYA

(*School of Textile Fibers, Kyoto Technical University*)

7,5,4'-Trimethoxymorin was synthesized by the partial methylation of morin. The procedure of this synthesis was as follows: morin was mixed with aluminium chloride ($Al_2Cl_6 \cdot 12H_2O$) in boiling pyridin solution, and thus obtained aluminium salt of morin was methylated with diazomethane in neutral methanol-ether solution. 7,5,4'-Trimethoxymorin was obtained in a crystalline form, after removing the combined aluminium with dilute hydrochloric acid from the methylated morin-aluminium salt. The crystal is pale yellow needle, melted at 226°C and gives greenish brown in color with ferric chloride or yellow in color with strong green fluorescence by the addition of aluminium chloride. It may be pointed out that the masking of 3,2'-hydroxy groups in morin with aluminium is involved in the process of this partial methylation.

Studies on Myrosinase. Part V. Relation between Myrosinase and Animal Myrosulfatase. (p. *1068～1071*)

By Zenji NAGASHIMA and Masaaki UCHIYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

Myrosulfatase has been considered as follows. It is distributed in plants (crucifers) and animal tissues and breaks the sulfuric acid ester-linkage of mustard oil glucoside. And in plants, myrosulfatase is present with thioglucosidase and these two enzymes constitute myrosinase which splits mustard oil glucoside into glucose, potassium bisulfate and mustard oil.

But the authors obtained the following results which showed that the animal myrosulfatase was different from the plant myrosulfatase.

(1) The animal myrosulfatase (prepared from *Chalonia lamps*) reacted on tetramethylsinigrin or tetraacetylsinigrin and broke their sulfuric acid ester-linkage. On the contrary, myrosinase of plant did not react on them. Namely, neither sulfuric acid ester-linkage nor thioglucoside-linkage of them was broken by myrosinase.

(2) Different from plant myrosulfatase, animal myrosulfatase was inhibited by NaF, Na_2SO_3 and so on. And animal myrosulfatase was activated by hydroxylamine and others while plant myrosulfatase was inhibited by them.

The authors consider that animal myrosulfatase is substantially different from plant myrosulfatase.

In the next paper, the authors conclude that myrosinase is not a mixture of two enzymes but a single enzyme which breaks the thioglucoside-linkage of the mustard oil glucoside, and that the sulfuric acid ester-linkage of the glucoside is spontaneously broken after the leaving of glucose.

When considered thus, the results of this experiments could be understood well.

On the Steamed Sweet Potato Product. Part VII. Determination of Sugars during the Manufacturing Process of Steamed Sweet Potato Product by the Ion Exchange Column Chromotography. (p. *1072～1076*)

By Shozo NARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Mie University*)

The amounts and species of sugar components of raw and steamed sweet potatoes were analized quantitatively by means of the ion exchange column chromatography.

Several percents of sucrose, glucose and fructose were the normal sugar components of raw sweet potatoes, but maltose was not detected at all. However, up to 20 to 30% of maltose (on day basis) was formed by steaming, while the amounts of sucrose, glucose and fructose were kept unchanged.

The amounts of these four sugars of the steamed and sliced products were not changed during further drying and storage processes, though the products began to separate a white powder on the surface and were covered completely by the thick white powder after about 3 weeks' storage.

The white powder thus formed was collected by a nylon brush from the surface of sliced sweet potato products (about 0.9% of the products), and was analyzed by the column chromatography.

The main component of the powder was maltose (85% on dry basis). A small amount of glucose, dextrin and starch (few percents each) were also found, but sucrose and fructose were not detected in the powder.

On the Steamed Sweet Potato Products. Part VIII. Relation between Contents of Sugars and Starch of Steamed Sweet Potato Products and their Quality. (p. *1077～1080*)

By Shozo NARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Mie University*)

Good quality slices are soft having brownish color, but poor quality slices are brittle.

Carbohydrate contents of both qualities were analysed by means of column chromatography and others. There was no difference between the amounts of glucose, fructose, sucrose and maltose of the two qualities, but the poor quality products contained about 30～40% of starch, while the good quality products contained only 10～25%.

Microscopic observations of slices of both products werh also made. Cells of poor quality slices were fullfilled with starch, and the cells were round in shape leaving intercellular spaces. On the other hand, cells of the good quality slices contacted each other as by the living cells, but the solid cellular contents were poor and were stained reddish violet with iodine.

Thus it was supposed that starch contents of raw sweet potato would define the quality of the steamed sweet potato products.

Biochemical Studies on the Growth of Sweetpotatoes. Part VII. Growth of Sweetpotatoes by Various Fertilization, the Inorganic Constituents of their Root Tubers and Corresponding Starches and their Photopastegraphy. (p. *1080～1087*)

By Shigeo SUZUKI, Taro TAMURA, Yoshiro NEMOTO and Katsusuke ARAI

(*Food Research Institute, Ministry of Agriculture and Forestry, Tokyo, Japan*)

Masatoshi ONODA and Yoichiro ÔTA

(*Kanto-tosan Agricultural Experiment Station*)

Growth of vines and root tubers of two varieties of sweetpotato, Norin No. 1 and Okinawa No. 100, were investigated under various conditions of fertilizer, together with their inorganic constitutents such as potassium, calcium, magnesium, phosphorus and iron. The characteristics of starch under various fertilization were also studied by means of starch photopastegraphy.

The following results were obtained.

1) The crude ash contents of root tuber increased with increase of potassium fertilizer. The rate of increase was larger in Okinawa No. 100 than in Norin No. 1. However, no significant effect of nitrogen fertilizer were observed both in Okinawa No. 100 and Norin No. 1 so far as crude ash contents is concerned.

2) Among inorganic constitutents of sweetpotato, potassium was predominant. Potassium content of Okinawa No. 100 was always larger than that of Norin No. 1. The amount of calcium, magnesium, iron and phosphorus were almost same with those two varieties.

3) The amount of potassium, calcium, phosphorus and iron of the corresponding starches separated from their root tubers were determined. Phosphorus was rich in Norin No. 1 and calcium also in Norin No. 1 except under potassium fertilizer lot. Amounts of potassium and phosphorus were found no significant difference.

4) By the photopastegraphy of those starches Norin No. 1 showed low gelatinization temperature and less swelling was found in the calcium rich starches.

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Biochemical Studies on the Rice Blast Disease. Part XV. Studies on the Differences of Pathogenicity among the Various Races of *Piricularia oryzae* CAVARA by the Infra-red Spectra. (Preliminary Report)

(p. *1089～1094*)

By Hitoshi OTSUKA

(*Faculty of Education*, *University of Niigata*)

The infra-red absorption spectra of mycelium of *Piricularia oryzae* CAVARA which grew on the various culture media and culture conditions, proved to have the reproducibility of a very high order. Under the same culture condition, infra-red absorption spectra of fifteen strains of *P. oryzae* were obtained. The comparisons of these infra-red spectra indicated that many strains have significant differences in their infra-red spectra. Each relative intensity of the 1540 cm^{-1} absorption against the 1660 cm^{-1} absorption was calculated, and each relative intensity was related with the author's classification of *P. oryzae* based on the biochemical differences.

The nucleoproteins were prepared from the mycelium of *P. oryzae*, and the infra-red absorption spectra of nucleoproteins of four strains were obtained. The comparisons of these infra-red absorption spectra indicated that four nucleoproteins have significant differences in 997 cm^{-1}, 1230 cm^{-1}, 1540^{-1} absorption. From the study of nucleoproteins extracts from mycelium *P. oryzae*, it has been found that the contents of nucleic acid in the nucleoprotein are closely related with the pathogenicity of *P. oryzae* and with the classification of *P. oryzae* based on the biochemical differences.

Studies on the Digestion of Proteins. Part I. On the Inhibitory Effect of Dopa-melanin on Trypsin.

(p. *1094～1096*)

By Sakae SHINANO and Goro TAMURA

(*Department of Agricultural Chemistry*, *Faculty of Horticulture*, *Chiba University*)

The effect of the inhibitory action of dopa-melanin on trypsin activity was studied and the results were as follows.

(1) The dopo-melanin greatly inhibited the activity of trypsin.

(2) The degree of the inhibitory action was greatly affected by the degree of preincubation or the time of incubation of the melanin solution with the enzyme before adding it to the substrate, and also by the sequence of mixing in the preincubation.

(3) The type of the reaction between dopa-melanin and trypsin was analysed by the method of Ackermann and Potter. From the shape of the curve which was obtained when the rate was plotted against the enzyme amount, it may be assumed that the reaction is not reversible.

(4) The type of inhibition was not competitive, nor uncompetitive type.

Studies on the Fermented Milk. Part III. Studies on Stabilizers of the Sour Milk Drink containing Natural Fruit Juice. (3) On the Methods for the Measurement of the Stability.

(p. *1097～1101*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co.*, *Ltd.*)

The stability of sour milk drink containing natural fruit juice was determined by the measuring the cloud or turbidity of the drink, both initially and after centrifugation, and by the storage test at room temperature and constant 37°C. These results indicate that (1) there is the correlation between the above cloud index and the stable period during the storage test at 37°C on the 1% significance level. (2) The ratio of the stable period (*R*) at room temperature and the stable period (*T*) at 37°C has no constant value due to the change of room temperature with seasons. But classified the drinks by the season of production into 3 groups, R/T ratio is near the constant and the mean value is 7.0 in winter, 5.3 in spring and 4.6 in summer. (3) R/T ratio of the drink containing propylen glycol alginate varies from lot to lot of P.G.A., but there is the correlation between *R* and *T* of the drink containing the same lot of P.G.A.

Studies on the Fermented Milk. Part V. Microbiologicol Studies on Yeasts isolated from Decayed Milk.

(p. *1102～1107*)

By Toyozo OOKI

(*Research Institute of Calpis Food Industry Co.*, *Ltd.*)

(1) One strain of yeast was isolated from decayed

milk. This yeast considerably resembles *Candida albicans* (Robin) Berkhout and *C. claussenii* Lodder et Kregar-van Rij. In morphological properties this yeast resembles *C. claussenii* and different from *C. albicans*. However the cells have not so long shape as those of *C. claussenii* and ring has not been formed in malt ext. Further, in physiological properties, the assimilation of saccharose, so characteristics of *C. claussenii*, has not been observed. Moreover neither the color change nor the coagulation of litmus milk has been observed.

This yeast, therefore, represents a separated species for which the author propose the name *Candida lactis* nov. spec.

(2) Properties of this yeast are following:

An ascospore has not been observed. In malt ext. cells are round to short oval $(4.6\sim7.7)\times(6.4\sim7.7)\mu$. True mycelium is not formed. *Pseudomycelium* is formed abundantly on the surface of the agar and rather primitive in the agar. Glucose, galactose, mannose, fructose and maltose are fermented and saccharose, raffinose, inulin, melezitose, α-methyl-D-glucoside, lactose, salicin and arbutin are not. Glucose, galactose and maltose are assimilated and saccharose, lactose and nitrate are not. Neither color change nor coagulation has been observed in litmus milk.

Studies on Changes in Chicken Egg Yolk during Storage. (p. *1108~1111*)

By Ryo NAKAMURA and Yasushi SATO

(*Department of Agriculture, University of Nagoya*)

About fresh and stored eggs, the protein compositions of their yolk were compared by three methods; these were electrophoretical, ultracentrifugal, and chemical analysis.

Electrophoresis was carried out by Tiselius electrophoretic apparatus using a Menzel's buffer (pH 9.65, μ 20.3). Ultracentrifuge was carried out by Spinco type E ultracentrifuge according to the method of Vandegaer et al. (J.E. Vandegaer et al., *Arch. Biochem. Biophy*. **62**, 328 (1956)). Chemical analysis was carried out to the separated components in egg yolk proteins.

The results of these analyses revealed that there was no change in the egg yolk proteins during storage, but concerning the lipid which was extracted by ether from yolk, there was a difierence in point of its amount and composition.

Studies on β-Amylase of *Wasabia wasabi*. Part I. Purification of the β-Amylase. (p. *1111~1115*)

By Aiko KASUGAI

(*Osaka Women's University*)

The tubers of wasabi (*Wasabia wasabi*), like sweet potato and soybean, were found to be an excellent source of β-amylase and hence the purification of the enzyme from wasabi was undertaken. The tubers were ground and suspended into a mixture of cysteine and ethylene-diaminetetraacetate and the pH of the supernatant solution was adjusted to pH 7.0 with diammonium phosphate. Then the enzyme solution was filtered and after being treated with anion exchange resin "Duolite A-2" in order to remove coloring matters, the solution was salted out with ammonium sulphate. After dissolving the enzyme obtained by salting-out into a dilute cysteine solution and dialyzing against frequent changes of distilled water, the enzyme was precipitated by adding alcohol to 40% by volume. The enzyme thus isolated was allowed to stand at room temperature and ammonium sulphate was added little by little when crystalline precipitates rich in β-amylase activity appeared in the solution.

Studies on β-Amylase of *Wasabia wasabi*. Part II. Enzymatic Properties of the β-Amylase. (p. *1115~1118*)

By Aiko KASUGAI

(*Osaka Women's University*)

Enzymatic properties of β-amylase of *Wasabia wasabi*, which was purified according to the method described in the preceding paper, were investigated. The enzyme was quite stable in a pH range from 4.3 to 8.2 at 17°C. But the enzyme was sensitive to heat and completely lost its activity at 60°C in five minutes. The pH optimum of the enzyme action was 5.4 and this was found to lie at rather alkaline side than that of the crude enzyme solution. The activity of the enzyme varied considerably with the kind of buffer used, just as reported by Ballou et all. (J. *Biol. Chem*., **139**, 233 (1941)). The degree of hydrolysis of soluble starch by the enzyme was about 30% as glucose and the paper-chromatograms revealed that the product were only maltose. The enzyme was easily inactivated by the addition of *p*-chloromercuri benzoate and the inactivated amylase was reactivated by cysteine. Thus the amylase, as well as β-amylase isolated from malt, sweet potato, soybean, etc. by other investigators, was concluded to be a SH-enzyme.

Studies on the Inhibition of Amylase Activity by Flavins. Part I. Inactivation of α-Amylase in the Presence of Riboflavin. (p. *1119~1123*)

By Kiyohide SONE

(*Department of Living Science, Faculty of Agriculture, Tohoku University*)

The relationship between riboflavin and α-amylase activity was investigated in vitro in this experiment. It was found that the photosensitized riboflavin by the illumination inhibits directly the dextrinizing power of enzyme, whereas it does not affect in the dark. The change of pH value of mixed solution of riboflavin and enzyme was not necessary for the inhibiting activity of riboflavin in both cases of light and dark. The inhibition by riboflavin on dextrinizing power was accelerated by the illumination of light especially by the longer wavelength than 680 mμ.

On the Viscosities of Starch Mash. Part I.

(p. *1123～1127*)

By Jiro KOBAYASHI, Chihiro KOBAYASHI and Takashi FUKINBARA

(*The Institute of Physical and Chemical Research*)

In order to research the hydrolysis of starch in cooking process, the relations between viscosities of starch mashes and cooking conditions were studied.

Sweet potato starch solutions were cooked as follows:

Temperature, 100°.

pH, 4.0 to 8.0.

Cooking time, up to 150 minutes.

Starch concentration (as glucose), 1 to 5 percent by weight.

The viscosities were measured by means of the capillary tube viscometer and the viscosity constants of starch were calculated as Pseudo-plastic and Bingham-plastic fluid.

The results were as follows:

(1) In cooking at pH 5.4 to 8.0 and time up to 60 minutes, each viscosity constant showed a constant value. Accordingly it was proved that the starch was not hydrolyzed in these conditions.

(2) At pH 4.0, each viscosity constant became lower up to 120 minutes and then reached to a certain definite value. Accordingly it was proved that the starch was hydrolyzed to a certain degree.

On the Viscosities of Starch Mash. Part II.

(p. *1127～1130*)

By Jiro KOBAYASHI, Chihiro KOBAYASHI and Takashi FUKINBARA

(*The Institute of Physical and Chemical Research*)

In the present paper the relations between the viscosities of starch mashes and cooking conditions at temperature 133° were studied by the same method in the previous paper.

The results were as follows:

(1) In cooking at pH 5.4 to 6.8, the viscosity constants became lower and then showed a certain definite value. Accordingly it was proved that the starch was hydrolyzed and then the degree of hydrolysis reached to a certain difinite value.

(2) At pH 4.0, the viscosity constants became rapidly lower and the starch mashes almost lost the rheological property and then mashes became Newtonian's fluid.

The iodine reaction showed blue color in spite of rapid decrease of viscosity constants, but the formation of a few reducing sugar was recognized by Bertrand's method.

Precipitation of Some Amino Acids in a Crystalline Form in Process of Miso Brewing.

(p. *1130～1133*)

By Shunro KAWAKISHI, Susumu HIRANO and Hisao YOSHII

(*Aichi Institute of Food Technology, Nagoya*)

It was well known that the white granulous material, "KIBI-TSUBU" was precipitated in a crystalline form in process of Miso brewing. To make clear the formation of the precipitates, as a preliminary experiment, the authors studied the amino acid composition of the precipitates by paper chromatographic and microbiological methods.

The results obtained were as follows:

(1) Leucine, isoleucine, valine, alanine, glycine, tyrosine, histidine and an unidentified amino acid were detected in the precipitates.

(2) The principal amino acids were leucine and isoleucine and they amounted to 60～70% of the total amino acids in the precipitates.

(3) Identification of leucine and isoleucine prepared from the precipitates was done by means of elementary analysis and IR-spectra method.

Comparison of α-Casein and Para-α-Casein. Part I. (Studies on Milk Coagulating Enzymes. XV)

(p. *1134～1138*)

By Kunio YAMAUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

α-Casein and para-α-casein were compared with respect to the coagulability by alkaline earth metal salts and by acid. Comparisons were also carried out on acid-base titration curve and calcium-binding property. The results were as follows.

(1) Solubility curve of para-α-casein changed from a gentle slope to a steep one against the abscissa of calcium chloride concentration with progress of rennin action. After completeness of the reaction para-α-casein precipitated at a critical calcium chloride concentration sharply. The critical concentration of various alkaline earth metal salts to coagulate para-α-casein increased in order of $BaCl_2 < CaCl_2 < SrCl_2 < MgCl_2$. Above about these critical concentrations α-casein became the state of opalescence.

(2) Acid coagulation region of para-α-casein was considerably wider than that of α-casein especially on the alkaline side of isoelectric point (acetate buffer; ionic strength 0.1). Solubility in urea solution of acid-coagulated para-α-casein was slightly lower than that of α-casein between 3 to 6 M urea concentration in the presence of 0.1 M sodium chloride.

(3) No significant difference of acid-base titration curve was detected except for the bent region between pH 9.2～10.2, where para-α-casein had slightly higher alkali-binding capacity.

(4) Comparison of calcium-binding capacity was carried out on unfractionated whole casein and paracasein by membrane equilibrium method. The difference between casein and paracasein seemed to be within experimental error.

Author concluded that coagulation of α-casein by rennin might not be attributed to the change of net charge or the calcium binding capacity as the whole of α-casein. Factors related should be rather searched in the special complexed composition and structure of α-casein.

Comparison of α-Casein and Para-α-Casein. Part II. (Studies on Milk Coagulating Enzymes. XVI)

(p. *1139～1144*)

By Kunio YAMAUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, University of Tokyo*)

α-Casein and para-α-casein (rennin-treated) were compared in several properties.

(1) There was no difference in titratable value of SH-groups and color intensity of diazo reaction, phenol reaction and biuret reaction. Difference was also undetectable as to wave length of maximum absorption in ultraviolet region at pH 6.4, 9.4 and 11.7. At pH 6.4 and 9.4 extinction of para-α-casein was somewhat higher than that of α-casein over the whole range measured. This difference, however, seemed to be attributed to the defference of turbidity. At pH 11.7 both spectra were completely identical.

(2) Difference of ultracentrifugal patterns was not detected at pH 11.7, where both α- and para-α-caseins dissociated into the state of monomer having a very symmetrical single peak of the almost same S value. If the samples were neutrized to pH 7.0 (in the state of polymer), however, the patterns of para-α-casein became more heterogeneous than that of α-casein.

(3) No difference was found in the rate of dephosphorylation of α- and para-α-caseins by prostate phosphatase. It seemed to be unlikely that change of phosphorus linkage had any essential bearing on the formation of para-α-casein.

Studies on Myrosinase. Part VI. Mechanism of Decomposition of Mustard Oil Glucoside by Myrosinase.

(p. *1144～1149*)

By Zenji NAGASHIMA and Masaaki UCHIYAMA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Shizuoka University*)

In order to discuss the present subject, some experiments were carried out resulting as follows.

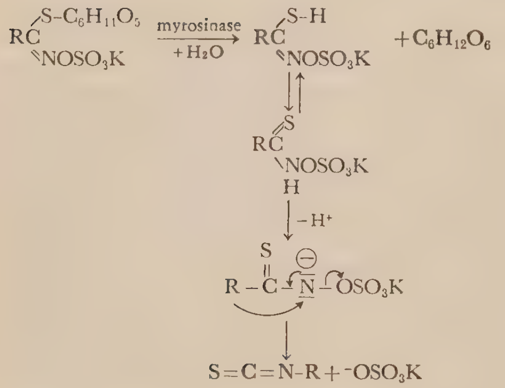
(1) Myrosinase reacted very slowly on merosinigrin and glucose was split from the substrate. But the formation of allylisothiocyanate was not recognized in this reaction.

(2) Against Sandberg's report, it was observed that glucose, sulfate and allylisothiocyanate were formed almost simultaneously, when myrosinase attacked on sinigrin.

(3) When treated $Na_2S_2O_3$ or NaCl, monosilversinigrate was converted to allylisothiocynate.

In previous papers the authors described the fair possibility that myrosinase was a single enzyme. Considering the results of the authors' experiments reported in parts I～VI, together with Ettlinger's, the authors conclude as follows.

Myrosinase is not a mixture of two separate enzymes as suggested by Neuberg, but a single enzyme. The enzyme hydrolyzes the thioglucoside-linkage of the mustard oil glucoside and consequently the sulfuric acid ester-linkage of the glucoside is spontaneously broken and mustard oil is formed. This mechanism is quite supportable from the view of electronic theory and the process is shown in the figure below.



Studies on Esterases from Seeds. Part V. Hydrolysis of *p*-Nitrophenyl Acetic Acid by Sulfhydryl Amino Acids.

(p. *1150~1154*)

By Tetsujiro OBARA and Yasokichi OGASAWARA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tokyo University of Education*)

It has been shown in a previous report that *p*-nitrophenyl acetic acid (*p*-NPA) is decomposed not only by the esterase itself but also by some substances in the crude solution. Furthermore, it has been observed that the hydrolysis of *p*-NPA may be accelerated by -SH amino acids.

Now, in order to probe more exactly these results we have examined the influence of cysteine, glutathione, cystine, methionine and vitamin B_1 on the hydrolysis of *p*-NPA.

In our experiments, the hydrolysis by such amino acids and vitamin B_1 was estimated by the ultraviolet absorption spectra.

The results of experiments mentioned above were as follows:

(1) The hydrolysis of *p*-NPA at pH 7.38 by the inactivated crude solution was greater than that at pH 7.0.

(2) The hydrolysis of the *p*-NPA was greatly accelerated by cysteine and glutathione containing -SH group, but was not accelerated by methionine, vitamin B_1 and cystine containing S or -S-S- group.

(3) The rate of the hydrolysis of *p*-NPA was linearly proportional to the logarithm of the concentration of -SH amino acids.

(4) There was the decrease in the hydrolysis of *p*-NPA by -SA amino acids with the decrease in the pH value of the solution.

Synthesis of Disaccharides by Mercuric Salt. Part III. Formation of 1,6-Glucosidic Linkage by the Reaction of 1,2-O-Isopropylidene-D-glucofuranose and 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl Bromide.

(p. *1154~1156*)

By Kazuo MATSUDA and Takeshi SEKIGUCHI

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

A considerable amount of 1,6-glucosidic linkage was formed during the reaction of 1,2-5,6-di-O-isopropylidene-D-glucofuranose (DAG) and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl bromide (ABG) with mercuric cyanide.

On the assumption that 1,6-glucosidic linkage was formed by the condensation of ABG and 1,2-O-isopropylidene-D-glucofuranose (MAG) which was produced by the partial decomposition of DAG, ABG and MAG was condensed with mercuric cyanide nearly in the same condition as the reaction of ABG and DAG. The reaction product, after removal of the protecting groups, was acetylated and fractionated by Magnesol : Celite column chromatography. 1,6-β-Linked gentiobiose was isolated and identified as its crystalline β-octaacetate.

Synthesis of Disaccharides by Mercuric Salt. Part IV. Synthesis of 3-O-D-Glucopyranosyl-D-glucose (Nigerose). (2) Reaction of 1,2,4,6-Tetra-O-acetyl-β-D-glucopyranose and 2,3,4,6-Tetra-O-acetyl-α-D-glucopyranosyl Bromide.

(p. *1156~1158*)

By Kazuo MATSUDA

(*Department of Agricultural Chemistry, Faculty of Agriculture, Tohoku University*)

For the direct proof of the structure of nigerose, 1,3-α-linked glucobiose was synthesized by the application of the Königs-Knorr reaction.

1,2,4,6-Tetra-O-acetyl-β-D-glucopyranose (I) and 2,3,4,6-tetra-O-acetyl-α-D-glucopyranosyl-bromide (II) were shaken in anhydrous nitromethane with mercuric cyanide. Residual (II) was removed by precipitation with benzylamine. The products, after removal of the acetyl group, were fractionated by carbon : Celite column chromatography. Nigerose and laminaribiose were obtained and characterized as their crystalline β-octaacetates.

Studies on Bacterial Amylase. Part XXVII. Formation of Bacterial Amylase. (X) Relationships between the Enzyme Formation and the Nucleic Acid Metabolism.

(p. *1159~1161*)

By Juichiro FUKUMOTO, Takehiko YAMAMOTO and Daisuke TSURU

(*Institute of Polytechnics, Osaka City University*)

The amylase formotion by washed cells of *Bacillus amyloliquefaciens* Fnkumoto was inhibited by the addition of base analogues of nucleic acid such as 8-azaguanine (8-AG) and thiouracil (TU), as has been reported previously. However, the degree of inhibition by the analogues was found to vary depending upon the kind of sugar used as the carbon source. The inhibition was severe when glucose or maltose was used while in the use of galactose or lactose only a slight effect was noticed. Also, it was found that the inhibitory effect of the analogues on amylase formation became significantly remarkable when an additional nitrogen source was present in the cell suspension system, regardless of the type of carbon source used.

Shaking the cells in the presence of 8-AG and carbon- and nitrogen source, led to formation of 8-AG-containing ribonucleic acid in the cells. On the other hand, the washed bacterial cells secreted several kinds of nucleic acid-bases in a free state, as reported previously. The amount of the bases secreted increased with the time of aerobic incubation of the cells but was considerably influenced by the type of carbon source, just as amylase formation was affected by this. Thus, the relationships between the enzyme formation and the nucleic acid metabolism were discussed.

On Polarography of 1-Deoxy-*p*-toluidino-I -fructose.

(p. *1162～1167*)

By Susumu ADACHI

(*Laboratory of Animal Products Technology, Faculty of Agriculture, Tohoku University, Sendai*)

1-Deoxy-*p*-toluidino-D-fructose (I) shows a single reduction wave in non-buffered solution in the range of pH between 3.5 and 12.7. In buffered or non-buffered solutions below pH 3.5, the catalytic hydrogen wave is given instead of the reduction wave of I. The limitting currents of 0.1 M $CaCl_2$ and 0.02 M LiCl solution are principally diffusion currents with the half wave potential of 1.580 and 1.978 volt, respectively, but the latter contains slightly the character of limitting current. The wave height in 0.1 M $CaCl_2$ solution pH 5 to 11 is constant and the half wave potential shifts to negative potential with the increasing pH. The wave height is proportional to the concentration of I in the range of 2.5×10^{-4} to 10^{-3} M. The temperature coefficient of the diffusion current in 0.1 M $CaCl_2$ solution is 1.30% at 25°. As the numbers of electron participating in the electrode reaction is 2, it may be reasonable to assume that the polarographic reduction product of I is 1-deoxy-*p*-toluidino-hexitol.

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